Cholesterol-lowering effects and potential mechanisms of different polar extracts from *Cyclocarya paliurus* leave in hyperlipidemic mice

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Abstract

Ethnopharmacological relevance: Cyclocarya paliurus Batal., native only to China, is widely consumed as a Chinese traditional folk medicine for the prevention and treatment of hyperlipidemia, obesity, and diabetes. The aim of the study is to investigate the cholesterol-lowering effect and potential mechanisms of different polar extracts from *Cyclocarya paliurus* leaves in mice fed with high-fat-diet.

Materials and methods: Cyclocarya paliurus leaves extracts were orally administered to diet-induced hyperlipidemic mice for 4 weeks. Simvastatin was used as a positive control. Body weight, food intake, histopathology of liver and adipose tissues, hepatic and renal function indices, lipid profiles in the serum and liver were evaluated. Total bile acid concentrations of the liver and feces were also measured. Furthermore, the activities and mRNA expression of cholesterol metabolism-related enzymes including 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase (CYP7A1) and acyl-CoA cholesterol acyltransferase 2 (ACAT2) in the livers of the mice were analyzed. LC–MS detection was performed to identify the components in the active fraction of *Cyclocarya paliurus* extracts.

Results: Different *Cyclocarya paliurus* polar extracts, especially ChE reduced the levels of serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and hepatic TC and TG, enhanced the level of serum high-density lipoprotein cholesterol (HDL-C), restored hepatic and renal function indices and histomorphology. HMG-CoA reductase activity and mRNA expression were decreased, while CYP7A1 activity and mRNA expression as well as the level of fecal and hepatic bile acid were increased by ChE. LC-MS analysis of ChE revealed the presence of six main triterpenoids, which might be responsible for its antihyperlipidemic bioactivity.

*Conclusion*s*:* Evidently ChE possesses the best antihyperlipidemic activity, and the cholesterol-lowering effect is at least partly attributed to its role in promoting the conversion of cholesterol into bile acids by upgrading the activity and mRNA expression of CYP7A1 and inhibiting those of HMG-CoA reductase to lower the cholesterol biosynthesis.

Key words: Cyclocarya paliurus; Hyperlipidemia; Cholesterol 7α-hydroxylase (CYP7A1); HMG-CoA reductase; acyl-CoA cholesterol acyltransferase 2 (ACAT2)

Chemical compounds studied in this article:

Arjunolic acid (PubChem CID: 73641); Cyclocaric acid B (CAS No.182315-46-4); Pterocaryoside B (CAS No. 168146-27-8); Hederagenin (PubChem CID: 258538); 3*β*, 23-dihydroxy-12-ene-28-ursolic acid (CAS No.125137-37-3); Oleanolic acid (PubChem CID: 10494).

1. Introduction

Hyperlipidemia, a disorder of lipid metabolism, is considered to be one of the major risk factors for cardiovascular diseases (CVD) such as atherosclerosis, myocardial infarction and stroke (Arsenault et al., 2009; Ma et al., 2012). Hyperlipidemia comprises a heterogeneous group of disorders with an elevation of total cholesterol (TC), triglyceride (TG) (Goldstein et al., 1973) , and/or a decreasing of high-density lipoprotein cholesterol (HDL-C) in the plasma concentrations (Beaumont et al., 1970). Elevated of blood lipid level, especially increased circulating levels of low-density lipoprotein cholesterol (LDL-C) and/or TG can accelerate CVD (Berry et al., 2012; de Lemos et al., 2010; Harchaoui et al., 2009). Therefore, modulating the dysregulation of lipid metabolism is thought to be an significant approach to slow or prevent the development of CVD (Derosa et al., 2006).

Currently, hyperlipidemia has been treated with hydroxymethyl glutarate coenzyme A (HMG-CoA) reductase inhibitors (statins), bile acid sequestrants, acyl-CoA cholesterol acyltransferase 2 (ACAT2) inhibitors (ezetimibe), fibric acid derivatives (fibrates) and niacin in clinical practice (Toth, 2010). Those synthetic drugs can pertinently descend the concentration of cholesterol or triglyceride. For instance, statins form the basis of care, but are not able to treat all aspects of dyslipidemia (Wierzbicki et al., 2012). Furthermore, the utility of these currently available anti-hyperlipidemia drugs are limited by their adverse effects, including liver and kidney dysfunctions, myopathy and rhabdomyolysis (Jain et al., 2007). However, traditional herbal remedies such as “Affinal Drug and Diet” functional nutrients have evolved from ancient healing system and enjoyed a remarkable resurrection, which may be due to their better biocompatibility with the human system (Urizar and Moore, 2003).

Cyclocarya paliurus (Batal.) Iljinsk (family Juglandaceae), native to China, is the sole species in its genus and grows in the highland of southern China (Shu et al., 1995). According to record in Zhong Hua Ben Cao, *Cyclocarya paliurus* is a traditional Chinese medicinal plant with Qingre and Jiedu efficacies. Based on the traditional Chinese medicine theory, phlegm-turbid stasis and dampness-heat

obstruction are two of the most important pathological factors associated with glycolipid metabolism disorders. Thus, *Cyclocarya paliurus* leaves have been traditionally used as drug formulation or food for the prevention and treatment of hyperlipidemia, obesity and diabetes (FU Xiangxiang, 2009; Leng, 1994; Xie and Li, 2001). Locally known as “sweet tea tree”, *Cyclocarya paliurus* leaves as health tea has been first approved by the United States Food and Drug Administration in China (Wang and Cao, 2007). A clinical observation reported that the sweet tea could reveal a two-way regulatory action on blood lipid level (Shen et al., 2002). Lipid-lowering effect of *Cyclocarya paliurus* leaves extract was suggestively related to the suppression of digestive lipase activity in lipid-loaded hyperlipidemic mice (Kurihara et al., 2003). Huang et al. demonstrated that polysaccharide isolated from *Cyclocarya paliurus* extract was responsible for the hypolipidemic effect in fat emulsions induced hyperlipidemia mice (Huang et al., 2011). However, our previous work indicated that *Cyclocarya paliurus* polysaccharide did not appear to be the active antihyperlipidemic constituent (Wang et al., 2013a). Therefore, the active chemical composition responsible for its antihyperlipidemic action as well as its possible mechanisms is not clear at present.

Hence, in this study, we investigated the antihyperlipidemic effect of different polar fractions of *Cyclocarya paliurus* ethanol extract prepared from chloroform, ethyl acetate, n-butanol and aqueous extracts to modulate lipid metabolism in HFD-induced hyperlipidemic mice. We characterized the modulatory effects of ethanol extract and four different polar fractions from *Cyclocarya paliurus* leaves on lipid metabolism and examined the principle active fraction and then discussed the possible antihyperlipidemic mechanisms.

2. Materials and methods

2.1 Experimental Animals and Ethics Statement

KM male mice (18-22 g) were purchased from Shanghai Super - B&K laboratory animal Corp. Ltd. [Certificate No. SCXK (HU) 2008-0113] and bred in our animal facility. The animals were kept in controlled conditions of 12/12 hrs light/dark cycle, temperature (24 ± 2°C), relative humidity (60 ± 10%) and water *ad libitum*. The care and treatment of these mice were maintained in accordance with NIH publication no. 85-23 (revised in 1996) on "Principles of laboratory animal care". The Institutional Animal Care and Use Committee (IACUC) of Southeast University approved the project (Permit Number: 20131105).

2.2 Drugs and Reagents

Simvastatin tablets (ST) was purchased from Yichang Changjiang Pharmaceutical Enterprise (Hubei, China). Oleic acid and Bovine serum albumin (BSA) were supplied by Sigma- Aldrich (Shanghai, China). DMEM was offered by Gibco-BRL, (Grand Island, NY, USA). ELISA kits for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase (CYP7A1) and acyl-CoA cholesterol acyltransferase 2 (ACAT2) detection were purchased from Senbeijia Bioengineering Institute (Nanjing, Jiangsu, China). Maxima First Strand cDNA Synthesis Kit for RT-qPCR, Maxima SYBR Green/ROX qPCR Master Mix (2X) was the product of Thermo Fisher Scientific Co., Ltd. The kits for the assay of TC, TG, HDL-C, LDL-C, Oil red O, alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (CRE), uric acid (UA) and total bile acid (TBA) were supplied by Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). All other Chemicals were of analytical grade.

2.3 Extraction and isolation

Leaves of *Cyclocarya paliurus* were collected from Nanjing Forestry University in July and authenticated by Prof. Min-Jian Qin from China Pharmaceutical University (Nanjing, Jiangsu, China), and a voucher specimen (No. L20100033) was

deposited in the herbarium of the university. The air-dried and powdered leaves (1 kg) were extracted with 80% ethanol (3×20 L, each 2 h). The combined ethanol extracts were concentrated to yield crude extract (101 g) under reduced pressure.

The crude extract (101 g) was dissolved in 2 L of distilled water. The suspension was then partitioned with chloroform, ethyl acetate, and n-butanol to provide chloroform extract (ChE, 45.6 g), ethyl acetate extract (EAE, 10.2 g), n-butanol extract (BuE, 19.3 g) and aqueous extract (AqE, 25.0 g).

2.4 Chemical analysis of Cyclocarya paliurus chloroform extract

ChE powder was diluted to 5 mg/ml with DMSO and Methanol. LC–MS analysis was performed using an Agilent 1260 Infinity HPLC system equipped with a UV detector and BrukerAmaZon SL MS (Sparta, New Jersey, USA) detection. The electrospray ion mass spectrometer was operated in the negative ion mode and scanned in the m/z range 0–1400. ESI was conducted using a needle voltage of 4.5 kV. Nitrogen collision induced dissociation was achieved in a nebulizer set at 17.0 psi with the nitrogen dry gas at a flow rate of 8.0 L/min; capillary temperature was 180°C. Grace AlltimaODS C18 reverse phase column (5 μm, 4.6 mm × 250 mm) was used throughout this study and the column temperature was set at 30°C. The flow rate was 1.0 ml/min and the injection volume was 10 ul. A binary solvent system consisting of solvent A (acetonitrile) and solvent B (water with 0.01% formic acid) was used as the mobile phase. The gradient program used was as follows: 0-15 min, 45% A; 15-25 min, 45% A to 52% A; 25-30, 52% A; 30-40 min, 52% to 55% A; 40-50 min, 55% A; 50-80 min, 55% A; 95% A for final washing and equilibration of the column for the next run.

2.5 Induction of hyperlipidemia in mice

For the development of hyperlipidemia, mice weighing 26 ± 2 g were fed with high-fat-diet (HFD), consisting of cholesterol 2%, lard 10%, yolk 10%, bile sodium 0.5%, standard diet 77.5% (w/w) (Pang et al., 2002). After 6 weeks of dietary manipulation, blood samples were collected from the orbital venous plexus under

mild anesthesia and centrifuged (Beckman Coulter Allegra 22R Centrifuge) at 3000 r/min for 15 min to separate serum. The mice with a serum cholesterol level > 3.62 mmol/L (140 mg/dl (Hirunpanich et al., 2006)) were used for study.

2.6 Experiment design

Animals were divided in 8 groups of 10 mice each as follow:

Group 1: normal control (NC), fed 0.2 mL/100 g/day of 0.5% sodium carboxyl methyl cellulose (CMC-Na).

Group 2: hyperlipidemia control (HC), fed 0.2 mL/100 g/day of 0.5% CMC-Na.

Group 3: hyperlipidemic mice treated with ethanol extract (EE, 1.5 g/kg) (Wang et al., 2013a).

Group 4: hyperlipidemic mice treated with chloroform extract (ChE, 1.5 g/kg).

Group 5: hyperlipidemic mice treated with ethyl acetate extract (EAE, 1.5 g/kg).

Group 6: hyperlipidemic mice treated with n-butanol extract (BuE, 1.5 g/kg).

Group 7: hyperlipidemic mice treated with aqueous extract (AqE, 1.5 g/kg) (Li et al., 2011).

Group 8: hyperlipidemic mice treated with simvastatin tablets (ST, 20 mg/kg).

Each extract was dissolved or suspended in 0.5% CMC-Na. The dietary treatments continued for the remaining days of the experiment. All mice were treated by gavage once a day for 4 weeks. ST, used as positive control, was given to mice by the same administration route.

2.7 Cell culture

L-O2 cells were supported by the China Pharmaceutical University and originally from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were grown to 80% confluence and then incubated in 0.1% BSA for 12 h. Subsequently, cells were washed and incubated in 0.1% BSA+1 mM oleic acid/DMEM for 24 h, then co-incubation with ChE (10, 20, 50 mg/L) or ST(10 μM) for 24 h. Eventually, the cells were subjected to oil-red O staining or TC and TG determination as reported (Guo et al., 2010).

2.8 Observe the general condition of the mice

Mental activity, fur condition, water intake and food intake of the mice were observed every day. Body weights and food intake were recorded once two weeks.

2.9 Analyses of serum samples

During the experimental period, blood samples were collected from the orbital venous plexus under mild anesthesia every two weeks. After 4 weeks' test drugs treatment, overnight-fasted animals were anesthetized with 10% chloral hydrate (intraperitoneally, 0.3 mL/100 g). Blood samples were collected from the orbital venous plexus and centrifuged at 3000 r/min for 15 min, and then the sera were stored at -80°C.

Serum lipids level indices of TC, TG, HDL-C, LDL-C; hepatic and renal function indices of ALT, AST, BUN, CRE and UA were measured using commercial assay kits according to the manufacturer's directions by an automatic biochemical analyzer (Roche Modular DP, Basel, Switzerland).

2.10 Analyses of related organs

Related organs of the heart, liver, kidney, fat, small intestine and large intestine were moved immediately and weighed after proper cleaning with saline. The viscera samples were stored at -80°C for further study.

2.11 Evaluation of hepatic TC and TG levels and enzymatic activity

100 mg liver tissue was homogenized (1:9, g/ml) in anhydrous ethanol, and then centrifuged at 2500 rpm for 10 min at 4°C to obtain supernatant, and then hepatic TC and TG levels were measured by automatic biochemical analyzer. Another 100 mg liver tissue was homogenized (10%, w/v) in PBS (PH=7.4), and then centrifuged at 3500 rpm for 10 min at 4°C to obtain supernatant. Activities of HMG-CoA reductase, CYP7A1 and ACAT2 in the liver were measured using ELISA kits according to the manufacturer's directions.

2.12 RNA preparation, cDNA synthesis and real-time RT-PCR

100 mg liver tissue was chopped into small pieces and homogenized with 1 ml Trizol reagent (Invitrogen) on ice. Total RNA was prepared according to the manufacturer's protocol. cDNA was synthesized with the iScript Synthesis kit and real-time quantitative PCR was performed in 20 μl reactions to detect the expression difference of HMG-CoA reductase, ACAT2 and CYP7A1 in hepatic tissues among treatments using SYBR-Green Quantitative PCR Kit. Amplification was performed with the ABI 7500 sequence detection system using the following protocol: 40 cycles (30 sec at 95°C and 30 sec at 60°C) after an initial activation step for 10 min at 95°C. *β*-actin was used as an internal control. Primer sequences are shown as follows:

β-actin [114 base pairs (bp)] forward primer: 5′- GCTCCGGCATGTGCAAAG-3′,
reverse primer: 5′- CCTTCTGACCCATTCCCACC-3′;

HMG-CoA (132 bp) forward primer: 5′-AGATAGGAACCGTGGGTGGT-3′,
reverse primer: 5′- TGCCACATACAATTCGGGCA-3′;

CYP7A1 (72 bp) forward primer: 5′- CCTCCGGGCCTTCCTAAATC-3′,
reverse primer: 5′- CACTCGGTAGCAGAAGGCAT-3′;

ACAT2 (138 bp) forward primer: 5′- ATTGTTGAAAGGTGGGCAGC-3′,
reverse primer: 5′- GGTAACATCCCATCCCGTCA-3′.

2.13 Determination of hepatic and fecal bile acid contents

Feces individually collected from last three days were dried by oven and stored at room temperature until analyses for bile acid contents. Liver and dry fecal samples were extracted with 90% ethanol (v/v) at 0 °C and then centrifuged at 2500 rpm for 10 min to obtain supernatant. The concentrations of bile acid were measured using a commercially available enzymatic kit according to the manufacturer's directions.

2.14 Histological evaluation of liver and adipose tissues

After the blood samples were gathered, liver and adipose tissues were collected and immediately fixed in neutralized 4% paraformaldehyde for 48 h at room

temperature. The samples were dehydrated through ascending grades of alcohol, cleared in benzene and, finally, embedded in low melting point paraffin wax. Sample sections (3 μm thick), were obtained by a rotative microtome and stained with HE for light microscopic examinations.

2.15 Statistical Analysis

Data are presented as means ± standard deviation. Differences were analyzed analysis of variance (ANOVA) followed by Dunnett's post-hoc test. For the analysis of body weight, food intake, TC and TG, repeated-measures-ANOVA was performed. $p<0.05$ was considered statistically significant.

3. Results

3.1 Phytochemical analysis

Identification of the components in ChE was established by comparison (retention times and mass fragmentation patterns) with commercial standards. There were six characteristic peaks in the HPLC fingerprint chromatogram of standards (Fig.1). As shown in table 1, the retention times and spectral data of the major peaks of chloroform extracts corresponds to that of standards. Arjunolic acid (RT: 18.7 min, $[M-H]^-$: 487.37), cyclocaric acid B (RT: 25.1 min, $[M-H]^-$: 485.38), pterocaryoside B (RT: 25.8 min, $[M-H]^-$: 621.46), 3*β*, 23-dihyreoxy-12-ene-28-ursolic acid (RT: 47.1 min, $[M-H]^-$: 471.37), hederagenin (RT: 48.1 min, $[M-H]^-$: 471.38) and oleanolic acid (RT: 76.6 min, $[M-H]^-$: 455.37) were detected in ChE.

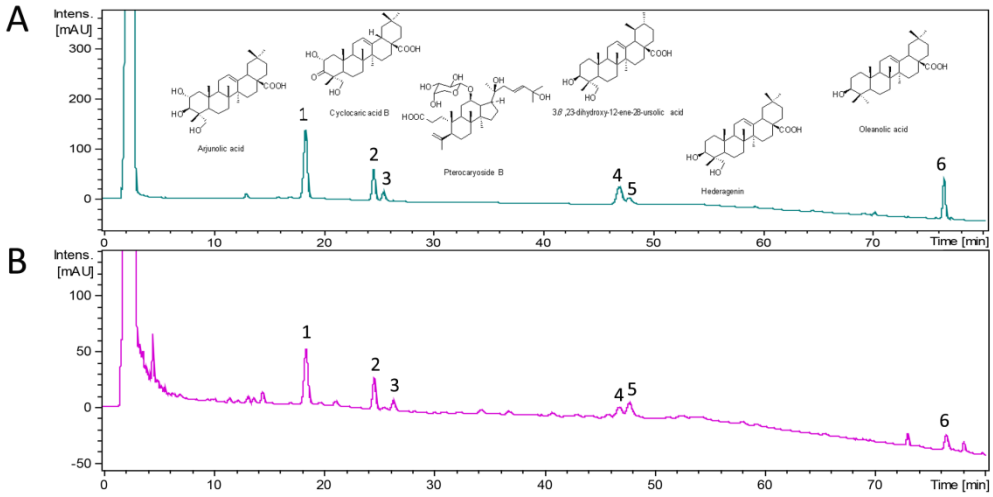

Fig. 1. HPLC analysis of standards (A) and *Cyclocarya paliurus* chloroform extracts (B) using UV detection.

Table 1 LC–MS/MS identification of compounds in chloroform extract of *Cyclocarya paliurus*.

Peak no.	Identification		Rt (min)	Molecular weight	$[M-H]^-$	MS^2
1	Arjunolic acid	ChE	18.7	488.70	487.37	409.23 (100); 421.22 (18); 379.22 (12)
		St.	18.7		487.39	409.20 (100); 421.22 (15); 379.21 (11)
2	Cyclocaric acid B	ChE	25.1	486.68	485.38	391.20 (100); 455.26 (28)
		St.	25.2		485.35	391.20 (100); 455.19 (27)
3	Pterocaryoside B	ChE	25.8	622.83	621.46	489.35 (100); 521.28 (70); 389.23 (42); 371.26 (15)
		St.	25.8		621.40	489.32 (100); 521.27 (73); 389.23 (39); 371.19 (15)
4	3*β*,23-dihydroxy-12-ene-28-ursolic acid	ChE	47.1	472.70	471.37	393.22 (100); 405.19 (23)
		St.	47.0		471.37	393.20 (100); 405.19 (31)

5	Hederagenin	ChE	48.1	472.70	471.38	393.25 (100); 405.19 (35)
		St.	48.1		471.36	393.21 (100); 405.29 (29)
6	Oleanolic acid	ChE	76.6	456.70	455.37	407.23 (100)
		St.	76.6		455.34	407.24 (100)

The characteristic fragmentation ions was showed at m/z (relative intensity %). ChE: *Cyclocarya paliurus* chloroform extract; St.: standard.

3.2 Body weight and food intake

During the entire feeding period, HFD-fed groups displayed a declined food intake compared with the NC group owing to the unpleasant taste and texture of HFD. After 6 weeks of HFD exposure, mice got an extraordinary gain of body weight. Treatment with the extracts did not affect food consumption, but showed a remarkable decrease on body weight; especially group EE, ChE and EAE (Table 2).

Table 2 Effect of different polar extracts on body weight and food intake of HFD induced hyperlipidemic mice

	Group / Week		NC	HC	EE	ChE	EAE	BuE	AqE	ST
Body weight (g)	Before administration	0	25.8±1.8	25.8±2.0	25.8±1.7	25.8±1.8	25.8±1.9	25.8±1.0	25.8±2.1	25.8±1.9
		2	29.6±1.8	28.1±1.8	28.4±2.1	27.9±2.5	27.5±3.1	27.7±2.1	28.0±3.1	27.3±0.9*
		4	31.0±2.3	32.0±1.8	31.1±0.7$^{\#\#}$	30.7±2.8$^{\#}$	30.0±2.6	31.0±1.6$^{\#\#}$	30.2±2.9	30.5±2.3$^{\#}$
		6	32.4±1.3	31.9±0.9	31.8±2.7	32.0±3.5	32.0±1.4	31.4±0.8	31.6±2.8	31.7±1.9
	After administration	1	34.2±1.0	32.3±2.8	29.7±2.3*	30.0±3.4	29.2±1.9**$^{\#}$	30.1±2.0*	29.5±2.4*	29.2±3.4
		2	33.4±0.7	32.2±2.9	28.8±2.9*$^{\#}$	28.1±3.0**$^{\#}$	28.4±2.4**$^{\#\#}$	28.6±3.1*$^{\#}$	28.1±2.2**$^{\#\#}$	28.5±3.4*$^{\#}$
		3	33.3±1.1	32.5±1.7	29.0±2.0**$^{\#}$	28.8±2.3**$^{\#}$	28.3±2.2**$^{\#\#}$	28.5±1.7**$^{\#\#}$	28.3±3.1*$^{\#}$	28.3±3.1*$^{\#}$
		4	33.0±1.0	32.1±1.0	27.7±2.1**$^{\#\#}$	26.9±2.0**$^{\#\#}$	26.7±2.3**$^{\#\#}$	28.9±2.5*$^{\#}$	28.9±2.9*$^{\#}$	28.5±2.6**$^{\#}$
food intake (g/day)	Before administration	2	38.5	22.7	24.5	23.2	20.1	20.4	23.2	23.6
		4	36	27.9	28.7	27.1	24.2	26.9	25.1	28.2
		6	37.7	27.7	26.4	24.4	26.1	28.9	29.7	26.7
	After administration	1	36.7	24.4	26.3	24.1	25.8	24.4	27.9	25.9
		2	34.1	20.6	21.1	25.4	23.2	22.1	23.9	26.6
		3	32	23.7	22.6	24.6	22.1	25.5	25.2	30.5
		4	37.7	22.9	20.9	20.8	22.4	23.4	20.8	28

Results are expressed as mean ± S.D. (n=6). Data were analyzed by repeated-measures-ANOVA followed by Dunnett's post-hoc test. *$p < 0.05$, **$p < 0.01$ compared with NC; #$p < 0.05$, ##$p < 0.01$ compared with HC.

3.3 Serum lipid profile

As shown in Table 3, a meaningful increase in TC and TG levels was found in mice fed with HFD. By the continuation of HFD treatment and administration of test

drugs up to 4 weeks, the lipid levels were significantly reduced. At the 28th day, supplementations with ChE, EE and EAE significantly reduced the level of serum TC, TG and LDL-C (Table 3 and Fig.2). Conversely, HDL-C levels were significantly increased in test drug groups, especially in ChE group, followed by EE and EAE groups (Fig.2).

Table 3 Effect of different polar extracts on serum TC and TG levels in HFD induced hyperlipidemic mice

Group / Week			NC	HC	EE	ChE	EAE	BuE	AqE	ST
TC (mmol/L)	Before administration	2	2.01±0.10	2.87±0.22**	2.87±0.11**	2.86±0.20**	2.86±0.22**	2.86±0.42**	2.84±0.37**	2.84±0.13**
		4	2.08±0.13	3.64±0.32**	3.62±0.41**	3.68±0.29**	3.68±0.31**	3.67±0.27**	3.63±0.31**	3.61±0.29**
		6	2.08±0.19	4.18±0.34**	4.18±0.15**	4.16±0.19**	4.16±0.22**	4.17±0.27**	4.17±0.23**	4.18±0.23**
	After administration	2	2.06±0.22	4.11±0.19**	3.23±0.36**$^{\#\#}$	3.07±0.29**$^{\#\#}$	3.56±0.42**$^{\#}$	3.83±0.27**	3.82±0.27**	3.23±0.21**$^{\#\#}$
		4	2.06±0.17	4.12±0.25**	2.78±0.35**$^{\#\#}$	2.42±0.06**$^{\#\#}$	3.04±0.24**$^{\#\#}$	3.78±0.29**	3.76±0.41**	3.18±0.10**$^{\#\#}$
TG (mmol/L)	Before administration	2	0.79±0.08	0.84±0.09	0.84±0.10	0.83±0.13	0.85±0.12	0.83±0.08	0.85±0.12	0.83±0.12
		4	0.75±0.16	0.92±0.17	0.91±0.15	0.92±0.15	0.93±0.12*	0.91±0.16	0.92±0.10	0.92±0.12
		6	0.78±0.06	1.01±0.13**	1.04±0.18**	1.01±0.22*	1.00±0.15**	1.05±0.16**	0.98±0.19*	0.99±0.15*
	After administration	2	0.75±0.10	1.07±0.13**	0.88±0.11$^{\#}$	0.84±0.16$^{\#}$	0.92±0.15*	0.94±0.19	0.93±0.21	0.90±0.11*$^{\#}$
		4	0.77±0.17	1.06±0.13**	0.84±0.10$^{\#\#}$	0.78±0.17$^{\#\#}$	0.86±0.12$^{\#}$	0.93±0.14	0.91±0.12	0.85±0.04$^{\#\#}$

Results were expressed as mean ± S.D. (n=6). Data were analyzed by repeated-measures-ANOVA followed by Dunnett's post-hoc test. *$p < 0.05$, **$p < 0.01$ compared with NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with HC.

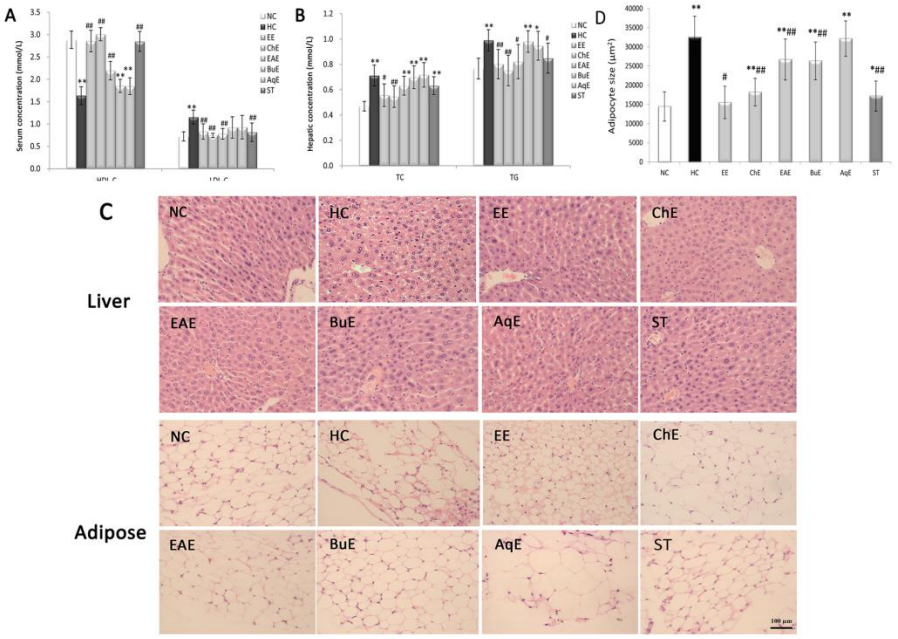

Fig.2. Effect of different polar extracts on serum HDL-C, LDL-C levels (A) and hepatic TC, TG levels (B) in HFD induced hyperlipidemic mice (mmol/L, n=6, mean ± SD). HE staining of liver and adipose tissues from different polar extracts treated in HFD induced hyperlipidemic mice (C). Results are expressed as mean ± S.D. (n=6). D, Effect of different polar extracts on adipocyte size (n = 3 per group. >150 cells were measured for each rat mean ± SD). Data were analyzed by ANOVA followed by Dunnett's post-hoc test. *$p < 0.05$, **$p < 0.01$ compared with NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with HC.

3.4 Histopathological analysis

As shown in Fig.2, normal hepatic architectural pattern was observed in NC group with centrilobular vein and portal triad well represented. In the liver sections of HC group infiltrating vacuolated cells were evident and large confluent lipid droplets could be observed around the centrilobular vein; the liver structure was seriously disorganized. After treatment with CP extracts or ST, the lobule revealed a reduction of degenerative lipid droplets around the centrilobular vein compared to hyperlipidemia controls. According to microscopic examinations, large cytoplasmic lipid droplets were found in the adipocytes from hyperlipidemic mice. The size of the adipocyte in HFD mice increased in comparison with NC group and was partly restored by the administration of CP extracts or ST, which was confirmed by the changes in adiposity indices and by cell size quantification.

3.5 Serum biochemical indices of hepatic and renal functions

Consuming orally different polar extracts as well as positive control significantly decreased all the hepatic and renal functional indices including ALT, AST, BUN, CRE and UA. ChE group showed the best modulation capacity, followed by EE and EAE groups. ST showed no significance in ALT, AST, BUN and CRE levels compared with HC group (Table 4).

3.6 Weight of related organs

The weight in most of the related organs was not affected by the supplement of each extract as shown in Table 4. However, ChE, EE and ST groups showed remarkably decreased in the weight of fat and ST group even had significant decrease in the weight of liver.

Table 4 Effect of different polar extracts on the activities of hepatic, renal functional indices and weight of related viscera samples in HFD induced hyperlipidemic mice

Group	NC	HC	EE	ChE	EAE	BuE	AqE	ST
hepatic and renal functional indices								
ALT (U/L)	22.00±6.57	51.50±9.40**	30.83±10.25$^{\#\#}$	27.83±6.08$^{\#\#}$	33.83±5.88**$^{\#\#}$	42.17±5.12**	43.50±9.89**	43.67±4.80**
AST (U/L)	100.33±8.07	167.83±16.80**	114.33±9.05*$^{\#\#}$	110.83±8.30$^{\#\#}$	127.83±9.77**$^{\#\#}$	148.83±12.43**	148.00±16.04**	151.50±7.37**
BUN (mmol/L)	5.26±0.81	8.30±0.60**	6.39±0.85*$^{\#\#}$	6.04±0.47$^{\#\#}$	7.40±0.36**$^{\#}$	7.75±0.31**	7.56±0.78**	7.71±0.37**
CRE (μmol/L)	9.50±1.05	14.83±0.75**	11.33±1.21*$^{\#\#}$	10.83±1.47$^{\#\#}$	11.67±0.52**$^{\#\#}$	13.67±1.63**	13.83±1.47**	13.50±1.87**
UA (μmol/L)	137.67±26.78	192.50±17.87**	168.67±15.24*$^{\#}$	158.67±23.40$^{\#}$	179.67±11.29**	177.33±13.00**	177.50±10.95**	169.67±10.37*$^{\#}$
Weight of related viscera samples (g/30 g body weight)								
Heart	0.13±0.01	0.14±0.01*	0.14±0.01	0.14±0.03	0.14±0.02	0.13±0.01	0.13±0.01$^{\#}$	0.13±0.01
Liver	1.34±0.06	1.39±0.07	1.46±0.16	1.42±0.19	1.36±0.06	1.43±0.09	1.31±0.11	1.22±0.08*$^{\#\#}$
Kidney	0.40±0.05	0.41±0.05	0.42±0.06	0.37±0.03	0.40±0.04	0.40±0.02	0.39±0.02	0.42±0.02
Fat	0.27±0.15	0.88±0.16**	0.51±0.14*$^{\#\#}$	0.40±0.12$^{\#\#}$	0.64±0.22**	0.74±0.16**	0.79±0.17**	0.30±0.11$^{\#\#}$

Results are expressed as mean ± S.D. (n=6). Data were analyzed by ANOVA followed by Dunnett's post-hoc test. *$p < 0.05$, **$p < 0.01$ compared with NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with HC.

3.7 Hepatic and fecal bile acid contents

The levels of hepatic bile acid and fecal bile acid were both significantly elevated in HC group compared to NC group ($p<0.05$). After 4 weeks administration, EE and ChE groups showed appreciably increased in fecal bile acid contents. Estimation of the hepatic bile acid contents in tested drug groups showed that EE and ChE groups had a considerable increase in excretion of bile acid (Fig.3). Hence, the increase in bile acid excretion contributed to the decrease cholesterol level in this study.

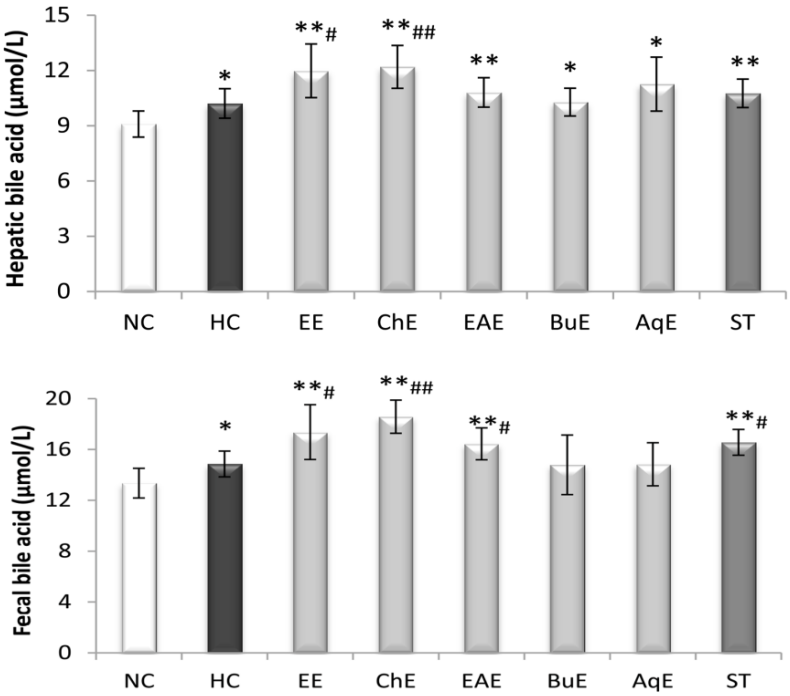

Fig. 3. Effect of different polar extracts on the activities of hepatic and fecal bile acid contents in HFD induced hyperlipidemic mice (μmol/L, n=6, mean ± SD). Data were analyzed by ANOVA followed by Dunnett's post-hoc test. *$p < 0.05$, **$p < 0.01$ compared with NC; #$p < 0.05$, ##$p < 0.01$ compared with HC.

3.8 TC and TG levels, enzymatic activities and gene expression in liver of mice

Hepatic TC and TG contents were significantly elevated in HFD fed groups as compared to those in NC group and consumption of ChE and EE dramatically decreased the hepatic levels of TC and TG (Fig.2). Experimental data presented in Fig.4 showed that the activity of HMG-CoA reductase in liver of hyperlipidemic mice was downregulated as compared with the control group ($p<0.05$), and further dramatically reduced in hyperlipidemic mice following administration of ChE and EE. On the contrary, the activity of CYP7A1 in liver of hyperlipidemic mice was up-regulated as compared with the control group ($p<0.05$), and further notably elevated following administration of ChE and EE. Otherwise, the activity of ACAT2 in liver of hyperlipidemic mice was remarkably elevated as compared with the control group ($p<0.05$), but no significant efficacy was observed following administration of ChE and EE. Real-time qRT-PCR data showed the same trend on gene expression of these enzymes in the liver as activities. ST did not show similar regulating effect on the activity and gene expression of CYP7A1.

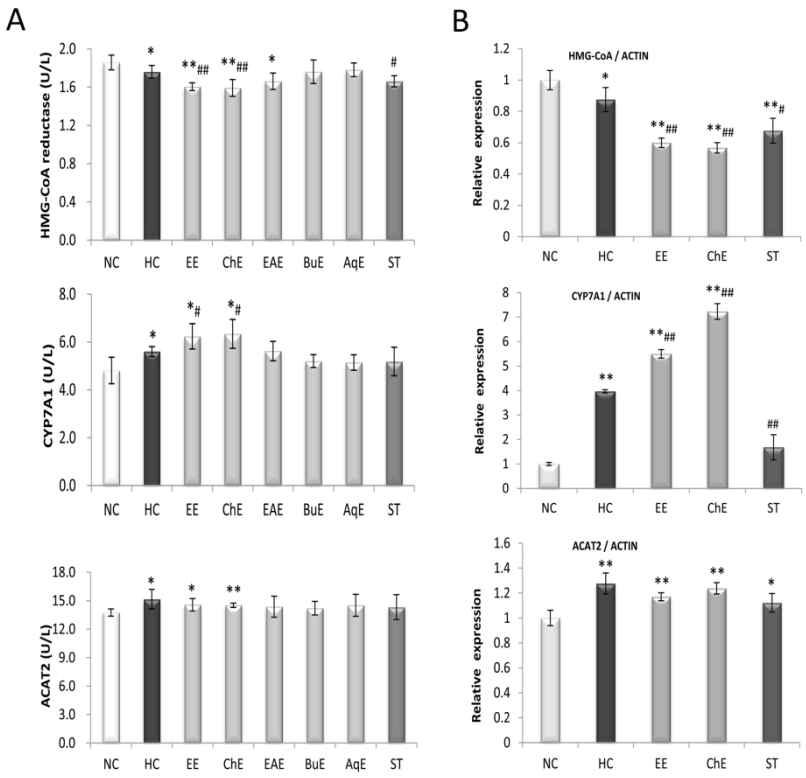

Fig. 4. Effect of extracts on the activities and mRNA expression of hepatic HMG-CoA reductase, CYP7A1 and ACAT2 levels in HFD induced hyperlipidemic mice (, n=6, mean ± SD). Data were analyzed by ANOVA followed by Dunnett's post-hoc test. U/L, enzyme activity in the supernatant fraction obtained from liver tissue homogenate (10%, w/v) in PBS. *$p < 0.05$, **$p < 0.01$ compared with NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with HC.

3.9 lipid-lowering effects in vitro

To verify the lipid-lowering effect, ChE incubated oleic acid induced hyperlipidemic cells for 24 h and tested its lipid-altering activities. As shown in Fig.5, oleic acid significantly increased fat deposition and cellular TC and TG content. After treatment with ChE, lipid accumulation in L-O2 cells was dramatically reduced as determined by oil-red O staining. Moreover, the intracellular content of TC and TG correspondingly decreased. All above data showed that ChE exhibited a predominant lipid-lowering activity in liver cells and it is reasonable to conclude that ChE is the active fraction accounting for the anti-hyperlipidemic effect of the *Cyclocarya paliurus*

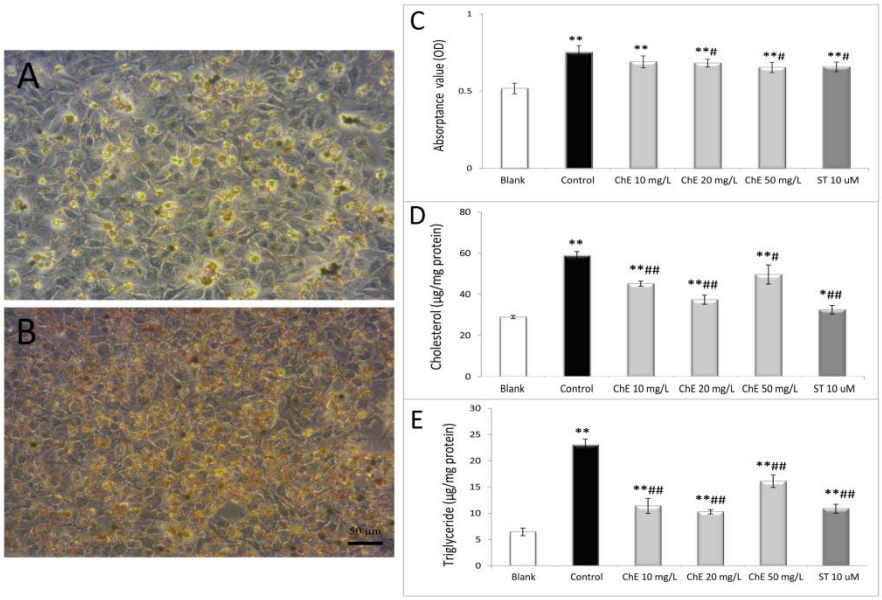

Fig. 5. Effects of chloroform extract on lipid profile in L-O2 cells. (A-B) The oil-red O staining pictures of normal and oleic acid induced hyperlipidemic cells. Cells were incubated in 0.1% BSA for 12 h. then cells were washed and incubated in 0.1% BSA+1 mM oleic acid/DMEM for 24 h, Subsequently, co-incubation with ChE (10, 20, 50 mg/L) or ST (10 μM) for 24 h. Eventually, the cells were subjected to oil-red O staining (C) or TC (D) and TG (E) determination. Data were analyzed by ANOVA followed by Dunnett's post-hoc test. $*p < 0.05$, $**p < 0.01$ compared with NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with HC.

4. Discussion

The present study showed cholesterol-lowering effect of different *Cyclocarya paliurus* polar extracts in HFD induced hyperlipidemic mice. Both serum and hepatic lipids could be modulated by different *Cyclocarya paliurus* polar extracts and ChE showed the best antihyperlipidemic activity. The underlying mechanism was approached by the characterization of interaction of different *Cyclocarya paliurus* polar extracts with the key enzymes in the process of cholesterol synthesis and metabolism. ChE might enhance the activity and mRNA expression level of CYP7A1 by promoting the cholesterol excretion of bile acid as its metabolite; meanwhile it inhibited the activity and mRNA expression level of HMG-CoA reductase to depress the cholesterol biosynthesis.

Hyperlipidemia refers to an elevation of lipids in the bloodstream and these lipids include fats, fatty acids, phospholipids, cholesterol esters, cholesterol, and triglycerides. Lipoproteins, such as chylomicrons, very-low-density lipoproteins, intermediate-density lipoprotein, low-density lipoproteins and high-density lipoproteins, are the combinations of proteins with cholesterol and triglycerides (Jain et al., 2007). HDL is considered to be “good” in the circulation; it, in fact, carries the cholesterol or cholesterol ester from peripheral tissues or cells to the liver for catabolism (Stein and Stein, 1999). LDL appears to play a key role in mediating the serum carriers of cholesterol to peripheral tissues (Jain et al., 2007). Those indicators for clinical diagnosis of hyperlipidemia were tested in the present study. A marked increase in serum TC, TG and LDL-C levels, along with a decrease in HDL-C level were observed in HFD induced hyperlipidemic mice. Regular administrations of different *Cyclocarya paliurus* polar extracts, especially ChE, modified the disorders of serum lipid metabolism. ChE consistently normalized serum TC concentrations in 4 weeks, which appears superior to ST. After the treatment of CP extracts, significant decreases were found in both TC and TG concentrations in vivo and in vitro.

The hypercholesterolemia is considered to be the most prevalent positive risk factor for initiation of atherosclerosis and coronary heart disease (Lewis, 2011). The metabolically active pool of cholesterol can be mainly derived from two sources: de

novo synthesis from acetyl CoA and uptake of sterol carried in LDL receptor (Dietschy et al., 1983). There are two main cholesterol outlets: first, through the activation of ACAT2, to combine the intracellular cholesterol and free fatty acids into cholesterol esters and stored in the cell. Second, excess cholesterol can be recycled or transported to the liver, generating bile acids, which are excreted with the bile into the intestine, finally eliminated into feces (Suckling and Stange, 1985).

Bile acids are synthesized in the liver and secreted into the small intestine where they facilitate cholesterol and fat absorption. Apart from the reabsorbed bile acids, the residue are excreted into feces (Lewis et al., 1995). Biliary secretion of cholesterol in the form of bile salts and phospholipids is of major importance for the lipid metabolism (Canbay et al., 2007). In the current study, fecal output of total bile acids was found to be enhanced in the groups treated with different *Cyclocarya paliurus* polar extracts, especially ChE, compared to the HC group. This is indicative of the potential of ChE to promote the conversion of cholesterol into bile acids in liver and to entrap bile acids in small intestine, which further enhances the excretion of bile acids from the feces, along with the fecal loss of steroids due to reducted enterohepatic recycling (Butt et al., 2007). These might be attributed to the possible mechanisms of the cholesterol-lowering effect of ChE on HFD-induced hyperlipidemic mice.

Cholesterol 7α-hydroxylase (CYP7A1), the first and rate-determining enzyme in the conversion of cholesterol to primary bile acids, catalyzes the initial step in cholesterol catabolism and bile acid synthesis and is mainly regulated by feedback inhibition of bile acids reabsorbed from the intestine (Pullinger et al., 2002). The enhancement in the excretion of bile acids seems to activate CYP7A1, increasing the conversion of hepatic cholesterol to bile acids for excretion (Chiang et al., 2001). This leads to a decreased concentration of hepatic free cholesterol, which may in turn stimulate LDL receptor expression and promotes the binding of LDL with the LDL receptor to lower blood cholesterol levels (Jain et al., 2007). Decreased blood and hepatic cholesterol in ChE intervention group might be attributable to the increased conversion of cholesterol to bile acids by increasing the activity and gene expression

of CYP7A1. Thus, development of a cholesterol-lowering drug that targets CYP7A1 may be a promising option with great prospects for lipid-lowering drugs.

HMG-CoA reductase is the rate limiting enzyme of hepatic cholesterol biosynthesis. Down-regulation of HMG-CoA reductase activity will lead to inhibit cholesterol de novo synthesis in the liver and thus reduce serum cholesterol level (Jurevics et al., 2000; Lee et al., 2003). Bile and cholesterol were reported to inhibit HMG-CoA reductase level and activity (Brown and Goldstein, 1986) and the significant variation in the HMG-CoA reductase activity and gene expression between the HFD and the control mice may relate to the absorption of exogenous cholesterol in this study. Statins, a currently most available potent cholesterol-lowering drug, were HMG-CoA reductase inhibitors with inhibition constant values in cholesterol biosynthesis, and subsequently, effectively lowered serum cholesterol levels in hypercholesterolemia (Istvan and Deisenhofer, 2001; Steinberg, 2008). Similar results were observed in the present study. Our study showed that the administration of ChE could significantly inhibit the activity and the expression of HMG-CoA reductase and reduce cholesterol level, which indicated that the cholesterol-lowering effect of ChE might be also due to the down-regulation of HMG-CoA reductase in the high-lipid diet induced hyperlipidemic mice.

Cyclocarya paliurus leave extract was reported to contain many phytochemicals constituents, including triterpenoids, polysaccharides, flavonoids, phenolic compounds, protein, etc. (Xie and Xie, 2008). Our study revealed that triterpenoids such as arjunolic acid, cyclocaric acid B, pterocaryoside B, hederagenin, 3*β*, 23-dihyreoxy-12-ene-28-ursolic acid and oleanolic acid are the major components of ChE. Lu *et al* found that total triterpenoid acid fraction from *Folium Eriobotryae* has a high anti-diabetic potential along with a good hypolipidemic profile (Lu et al., 2009). Furthermore, Kim et al. proved arjunolic acid isolated from *Campsis grandiflora* K. Schum. showed a significantly ACAT inhibitory activity (Kim et al., 2005). Ursolic acid, a pentacyclic triterpenoid found in many herbs and spices, could decrease lipid accumulation in the liver (Somova et al., 2003) and improve the hypolipidemic efficacy (Wang et al., 2013c). Similarly, oleanolic acid was reported to decrease serum

TC, TG, LDL and FFA, increased serum HDL in Lepdb/db obese diabetic mice (Wang et al., 2013b). In addition, other triterpenoids such as echinocystic acid, reinioside C and α, β-amyrin ameliorated the hyperlipidemia through systemic mechanism (Han et al., 2014; Li et al., 2008; Santos et al., 2012). Thus, the observations reported in this study suggest that the antihyperlipidemic effects of ChE are attributed to triterpenoids.

5. Conclusion

In summary, this study demonstrated that ChE appeared to be the best antihyperlipidemic ingredients among different *Cyclocarya paliurus* polar extracts in HFD induced hyperlipidemic mice. The potential cholesterol-lowering mechanisms of ChE could at least partly be attributed to the fact that it promoted the cholesterol conversion into bile acids by upgrading the activity and gene expression of CYP7A1 and inhibited the activity and gene expression of HMG-CoA reductase to depress the cholesterol biosynthesis. Thus, these promising findings indicated that ChE had great potential usefulness as a natural agent for treating hyperlipidemia.

Acknowledgments

This work was partially supported by grants awarded from the National Natural Science Foundation of China (No. 81503316, 81001379, 31270673), the Ninth Batch of "Six Talent Peaks" Project of Jiangsu Province (No.2012-YY-008), the Fundamental Research Funds for the Central Universities" (No.ZJ14103), and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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