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ARTICLE

Effects of Pu-erh Tea Aqueous Extract (PTAE) on Blood Lipid Metabolism Enzymes

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Disorders of blood lipid metabolism are primary risk factors for many diseases. Recently, the effects of pu-erh tea on blood lipid metabolism have received increasing attention. However, the mechanism underlying its ability to regulate blood lipid metabolism is unclear. We set out to study this through assessing the effects of pu-erh tea aqueous extract (PTAE) on the central enzymes of blood lipid metabolism, including lipoprotein-associated phospholipase A2 (Lp-PLA₂), lecithin: cholesterol acyltransferase (LCAT), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and pancreatic lipase (PL). We find that the Lp-PLA₂, HMGR and PL activities are inhibited by PTAE in dose-dependent manners and that the LCAT activity tends to increase with increasing PTAE concentrations. Lineweaver-Burk plot analysis reveals that PTAE acts as a competitive inhibitor for HMGR and PL and as a noncompetitive inhibitor for Lp-PLA₂. Moreover, we determine that its active ingredients include catechins, gallic acid, caffeine, free amino acids, and soluble sugar. However, the effect of each ingredient and whether any of them have synergistic effects are still unknown. The results suggest that pu-erh tea has a potent ability to regulate blood lipid metabolism and knowledge of the mechanisms provides insights into its potential therapeutic application as an alternative hypolipidemic drug.

Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze], one of the most popular beverages worldwide, has been considered to be a crude medicine for 4,000 years,¹ and it can be categorized into the following three types according to the manufacturing process:² 3: non-fermented tea (green tea), semi-fermented tea (oolong tea), and completely fermented tea (black tea and dark tea). Pu-erh tea is manufactured mainly in the Yunnan province of China and is consumed widely in southeast Asia. This tea undergoes a post-harvest fermentation stage before drying and steaming, during which microorganisms play very important roles in producing the taste, color, fragrance, and functional components. In recent years, pu-erh tea and its components have received considerable attention due to their potential human health benefits, such as mediating the metabolism of blood lipids,^{1, 4, 5} lowering the risk of cardiovascular diseases (CVDs)⁶ and cancers⁴, reducing serum lipoprotein,⁵ total cholesterol (TC) and triglycerides (TGs) levels,⁷⁻⁹ increasing the serum level of high-density lipoprotein cholesterol (HDL-C), and reducing the level of low-density lipoprotein cholesterol (LDL-C),⁷ as well as exerting

hypoglycemic,² free radical-scavenging,³ antioxidative,^{10, 11} antimutagenic and antimicrobial effects¹². The chemical compositions of tea include phenolic compounds (mainly catechins), oxidized phenolic compounds including theaflavins (TFs), thearubigins (TRs) and theabrownins (TBs), proteins, amino acids, carbohydrates, minerals and trace elements, trace amounts of lipids, sterols, vitamins, xanthic bases, pigments and volatile compounds.¹³

Recently, the effects of the dietary components on blood lipid metabolism have received increasing attention, including the vegetable proteins, dietary fiber and essential oils.¹⁴ Generally, blood lipids contain lipoproteins, TG, TC, HDL-C, LDL-C and free fatty acids. Dyslipidemia is associated with many diseases, such as CVDs, hyperlipidemia, obesity, hypocholesterolemia and coronary heart disease, most of which are great threats to human health. In this study, we examine four important enzymes involved in blood lipid metabolism, including (i) 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), a rate-controlling enzyme involved in the biosyntheses of cholesterol and other isoprenoids;¹⁵ (ii) lipoprotein-associated phospholipase A2 (Lp-PLA₂), also referred to as platelet-activating factor-acetylhydrolase (PAF-AH), which is involved in the oxidation of LDL-C;¹⁶⁻¹⁸ (iii) lecithin: cholesterol acyltransferase (LCAT), which plays a dual role in reverse cholesterol transport by maintaining a concentration gradient for cholesterol flux from cells to HDLs through the esterification of HDL-free cholesterol to cholesteryl esters and by maintaining HDL acceptor particle shape and structure,

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which is likely necessary for efficient reverse cholesterol transport;¹⁹ and (iv) pancreatic lipase (PL), a key enzyme involved in the digestion of dietary TGs that is responsible for the hydrolysis of 50–70% of total dietary fats.^{20–22}

Animal experiments and cell cultures have shown that pu-erh tea and its components have been consistently associated with improvement of blood lipid metabolism. But few studies have focused on the mechanism underlying its ability to regulate this process. Because of the increased evidence indicating the health benefits of pu-erh tea, further investigation is necessary to elucidate this mechanism. In this study, we set out to study this through assessing the effects of pu-erh tea aqueous extract (PTAE) on the central enzymes of blood lipid metabolism, including Lp-PLA₂, LCAT, HMGCR and PL. Moreover, the regular components of PTAE will be determined.

Results

Contents of PTAE

The chemical compounds in the PTAE from Menghai Tea Factory were analyzed, and the active ingredients, including catechins, gallic acid, caffeine, amino acids, and soluble sugar, were detected, which are shown in Table 1. Five major catechin forms (catechin, C; epicatechin, EC; epigallocatechin, EGC; epicatechin gallate, ECG; and epigallocatechin gallate, EGCG) were detected in the PTAE. The major tea polyphenols of green tea are catechins, which account for 73% (w/w).²³ Several studies have shown that catechins are responsible for most of the beneficial health effects of green tea.^{24–26} EGCG, the major active catechin, has been shown to reduce plasma cholesterol levels and liver cholesterol accumulation.^{27, 28} In this study, gallic acid (43.48%) is the most abundant compound

in the PTAE. Previous study has demonstrated that gallic acid has antioxidant activity at lower concentrations and that the addition of gallic acid to the diet decreases body weight gain, liver and adipose tissue weights, serum parameters and hepatic steatosis.²⁹ Besides, the PTAE is also found to contain a small amount of catechins (12.51%) and EGCG (0.98%), suggesting that catechins are probably the active components in the extract. Caffeine (4.86%) is another important component of the PTAE. Catechins and caffeine are shown to be synergistic with regard to their anti-obesity activities as well as their effects on hepatic and serum TC and TG levels.³⁰ Previous study has also demonstrated that the inhibitory effects of fat accumulation resulting from the combination of at least 2 of the components catechins, EGCG, and caffeine are stronger collectively than their corresponding effects alone.³¹ As a post-fermented tea, the manufacture of pu-erh ripe tea includes natural fermentation and prolonged storage.³² During the fermentation process, polyphenoloxidase catalyzes the oxidation of catechins, forming TFs, TR and TBs;³³ however, these oxidized phenolic compounds are barely detected in our study. It has been reported that the contents of flavonoid, ascorbic acid, and total catechins are lower in water extract of pu-erh tea than in that of other teas.¹⁰ The water extract of pu-erh tea showed marked antioxidant activity against lipid and non-lipid oxidative damage, strongly suggesting that some active substances responsible for the antioxidant activity of this extract may be formed during fermentation.¹⁰ However, they were unable to identify the active compounds. In our study, we observe that the contents of gallic acid, catechins and caffeine are 43.48%, 12.51% and 4.86% in the PTAE, respectively, suggesting that these compounds may be the major active components in this extract.

Table 1. Description of regular chemical components of PTAE.

Components	Total catechins	C	EC	EGC	ECG	EGCG
Content (%)	12.51 ± 0.34	0.98 ± 0.02	3.31 ± 0.02	6.67 ± 0.04	0.57 ± 0.01	0.98 ± 0.03
Components	gallic acid	Caffeine	Amino acids	Soluble sugar		
Content (%)	43.48 ± 0.62	4.86 ± 0.05	3.12 ± 0.03	10.55 ± 0.16		
Components	TFs	TRs	TBs			
Content (%)	ND ^a	ND ^a	ND ^a			

PTAE was extracted from pu-erh tea with boiling water and separated by gradient elution. By assessing the regular components of PTAE, we determined that its active ingredients were catechins, gallic acid, caffeine, free amino acids, and soluble sugar. The percentages represent values per 100 g of PTAE. Each value represents the mean ± SD (n=3).

^a ND: not detected.

Effect of PTAE on Lp-PLA₂ activity

The inhibition of Lp-PLA₂ by PTAE is shown in Figure 1A. PTAE decreases Lp-PLA₂ activity in a dose-dependent manner compared with the control group. The enzymatic activity of Lp-PLA₂ is almost completely inhibited by 1.65 mg/mL PTAE, which has an IC₅₀ of 0.40 mg/mL as calculated from the inhibition curve. The inhibition patterns associated with PTAE were examined, and as shown by the Lineweaver-Burk plot in Figure 1B, a change is observed in the slope and y-intercept of the curve but not in the x-intercept in the presence of an inhibitor. Kinetic analysis reveals a significant and dose-dependent decrease in the apparent V_{max} of 2-thio PAF. In contrast, the K_m values for 2-thio PAF is unaltered by the presence of PTAE. Thus, the mechanism of inhibition is clearly noncompetitive. PTAE results in linear noncompetitive inhibition with the 2-thio PAF as a variable substrate. The apparent inhibition constant (K_i) is determined from the plot of the vertical intercepts of the slopes against the PTAE concentration (Figure 1C). The intersection point on the inhibitor concentration axis of the plot reveals a K_i of 0.32 mg/mL.

Effect of PTAE on HMGR activity

The inhibition of HMGR activity by PTAE is evaluated by measuring the 1 min uptake of NADPH (2.65 mmol/L) in the presence of increasing concentrations of PTAE (0-0.9 mg/mL). As shown in Figure 2A, PTAE completely inhibited the uptake of NADPH in a concentration-dependent manner. The IC₅₀ of PTAE is 0.39 mg/mL, as determined from the least-squares regression line of the plot of the sample concentration (mg/mL) versus HMGR activity (%). The Lineweaver-Burk plot (Figure 2B) constructed from this analysis reveals that PTAE acts as a competitive inhibitor for the NADPH binding site of HMGR. The inhibition constant K_i obtained from the plot of the slope from the primary graph versus the PTAE concentration is 0.33 mg/mL (Figure 2C).

Effect of PTAE on LCAT activity

We illustrate the effect of PTAE on the enzymatic activity of LCAT in Figure 3. The enzymatic activity of LCAT in the presence of PTAE is higher than that of the control. LCAT activity in the presence of PTAE at a concentration of below 0.76 mg/mL is approximately 1.3 times higher than that of the control group, but this difference is not significant. However, PTAE greatly increases LCAT activity in a dose-dependent manner at concentrations of higher than 0.76 mg/mL.

Effect of PTAE on PL activity

The inhibition of PL activity by PTAE is shown in Figure 4A. PTAE inhibits PL activity in a dose-dependent manner. The addition of 0.20 mg/mL PTAE to the standard assay mixture results in a decrease in the rate of the 4-methylumbelliferone (4-MU) reaction by 50%. Virtually no 4-MU is released at concentrations of above 2 mg/mL. To determine the mode of inhibition of PL activity by PTAE, the velocities of the PL-catalyzed reaction in the presence or absence of various concentrations of substrate are measured. With 4-

methylumbelliferyl oleate (4-MU oleate) as the variable substrate, Lineweaver-Burk plot analysis (Figure 4B) reveals that PTAE acts as a competitive inhibitor for PL. The K_i value of PTAE for PL activity as determined from the plot of the slopes from the primary graph versus the PTAE concentration is 0.10 mg/mL (Figure 4C).

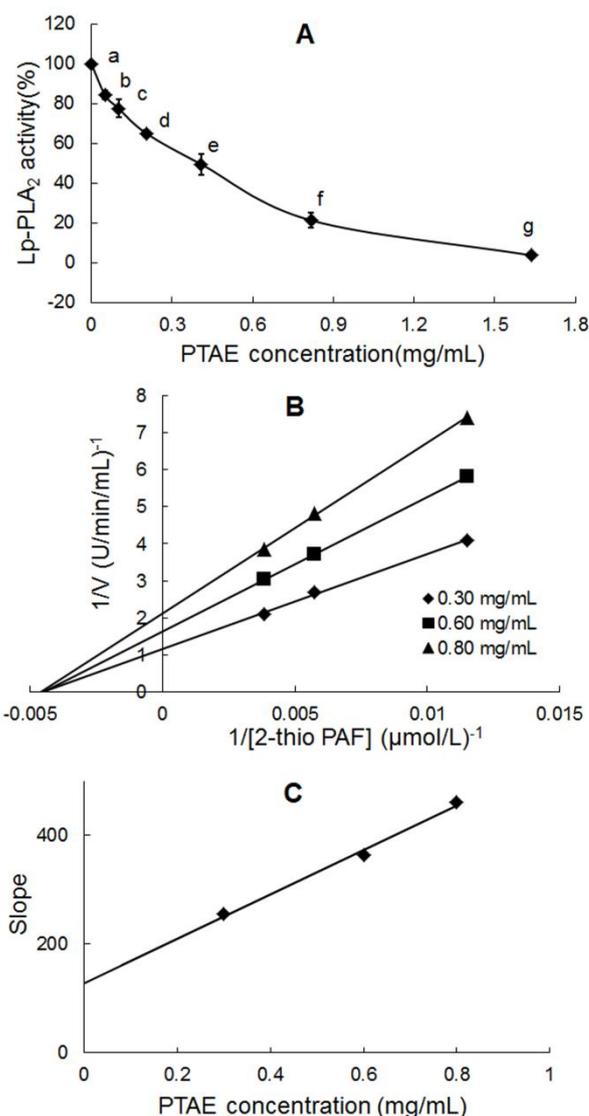


Figure 1. Dose-response effect of PTAE on Lp-PLA₂ activity *in vitro* and characterization of the inhibition. (A) Standard Lp-PLA₂ was incubated with 86.96 μM 2-thio PAF and treated for 20 min with PTAE (final concentrations 0, 0.05, 0.10, 0.20, 0.40, 0.80, and 1.60 mg/mL). The value of the absorbance change per minute was measured with a Model 680 Microplate Reader at 405 nm. The percent change caused by PTAE was expressed relative to the measurement of the 0 mg/mL PTAE-treated group, which was defined as 100%. Each value is the mean of triplicate experiments; (B) Lineweaver-Burk plot of the inhibition of Lp-PLA₂ activity by PTAE; (C) K_i determination for PTAE. Slopes from the Lineweaver-Burk plots (B) were plotted against the concentration of WSPPT.

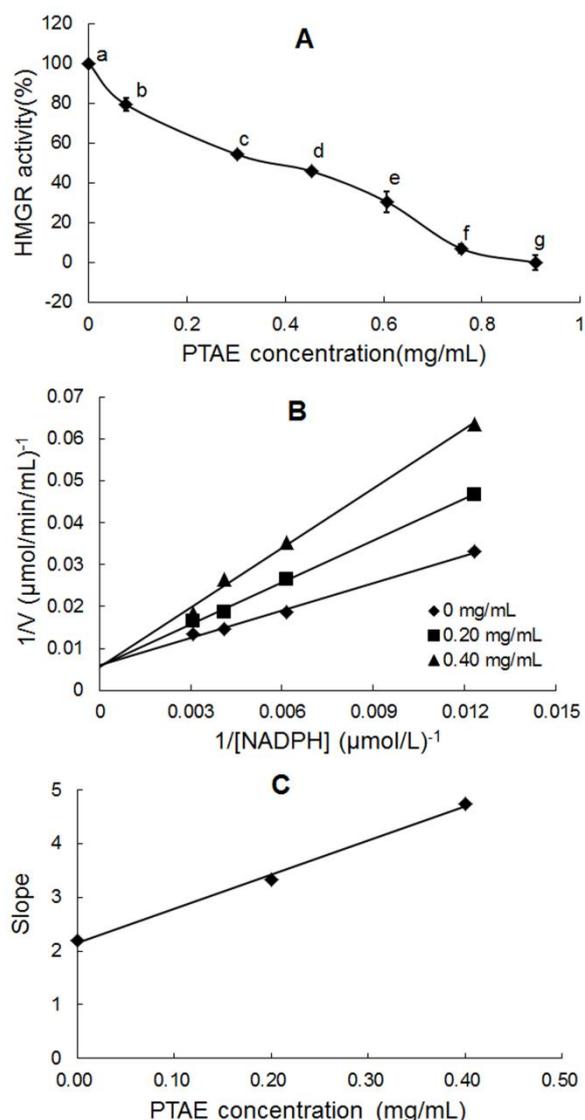


Figure 2. Dose-response effect of PTAE on HMGR activity *in vitro* and characterization of the inhibition. (A) A crude HMGR enzyme extract was incubated with HMG-CoA (465 μM) and NADPH (2.65 mM) and treated for 60 min with PTAE (final concentrations: 0, 0.08, 0.30, 0.45, 0.60, 0.70, and 0.90 mg/mL). The amount of NADPH taken up by HMGR was measured with a UV-VIS spectrophotometer at 340 nm. The percent change caused by PTAE was expressed relative to the measurement of the 0 mg/mL PTAE-treated group, which was defined as 100%. Each value is the mean of triplicate experiments; (B) Lineweaver-Burk plot of the inhibition of HMGR activity by PTAE; (C) K_i determination for PTAE. Slopes from the Lineweaver-Burk plots (B) were plotted against the concentration of PTAE.

Discussion

In this study, it is demonstrated that the chemical compounds in PTAE include total catechins (12.512%), gallic acid (43.38%), caffeine (4.86%), amino acids (3.12%) and soluble sugar (10.55%), of which catechins, gallic acid and caffeine have

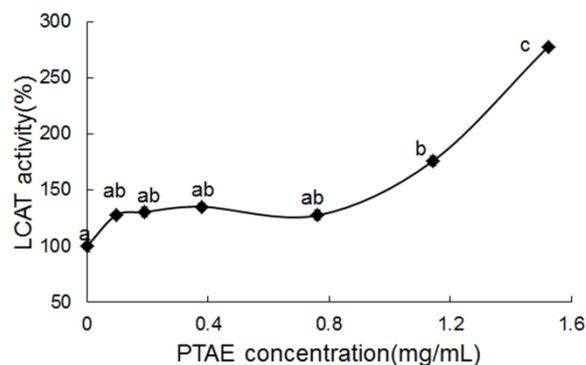


Figure 3. Dose-response effect of PTAE on LCAT activity *in vitro*. A crude LCAT enzyme extract was incubated with PTPB and treated for 20 min with PTAE (final concentrations: 0, 0.01, 0.02, 0.76, 1.14 and 1.52 mg/mL). The formation of *p*-nitrophenoxide in the reaction was monitored with a UV-VIS spectrophotometer at 400 nm. The percent change caused by PTAE was expressed relative to the measurement of the 0 mg/mL PTAE-treated group, which was defined as 100%. Each value is the mean of triplicate experiments.

positive effects on reducing the plasma cholesterol level and liver cholesterol accumulation.^{27, 29} Therefore, these components are probably the major active ingredients in PTAE, especially gallic acid, which is the most abundant component.^{29, 34}

Previous studies have shown that pu-erh tea significantly lowers the levels of blood lipids,^{2, 5, 7} including TG, TC, and LDL-C, although the underlying mechanism has not been determined. To further understand the mechanism involved in the effects of pu-erh tea on blood lipid metabolism, lipid-regulating enzymes, such as Lp-PLA₂, HMGR, LCAT, and PL, are investigated in this study. We demonstrate that PTAE reduces Lp-PLA₂, HMGR and PL activities in a dose-dependent manner compared with the control group without PTAE treatment and that it elevates LCAT activity. The IC₅₀ values for inhibition are 0.40 mg/mL, 0.39 mg/mL and 0.20 mg/mL for Lp-PLA₂, HMGR, and PL, respectively.

Lp-PLA₂ is a calcium-independent serine lipase associated with LDL particles in the plasma, and Lp-PLA₂ activity is correlated with total cholesterol ($r=0.52$, $n=126$) and LDL cholesterol ($r=0.60$, $n=126$) concentrations.³⁵ Several inhibitors of Lp-PLA₂ have been studied, including statins,³⁶ darapladib,^{16, 37} SB-222657 and SB-244323.³⁸ However, these inhibitors either have detrimental effects on normal lipid profiles or have poor aqueous solubility; thus, it is necessary to search for less lipophilic inhibitors. Pu-erh tea, a natural beverage, has been shown to significantly lower LDL-C and TG levels and to elevate HDL-C level in a rat hyperlipidemia model.⁶ However, little research has been conducted on its influence on Lp-PLA₂. This study is the first to demonstrate that PTAE causes a clear dose-dependent reduction in Lp-PLA₂ activity with an IC₅₀ of 0.40 mg/mL. PTAE acts as a noncompetitive inhibitor for Lp-PLA₂ with a K_i of 0.32 mg/mL. Moreover, it shows good water

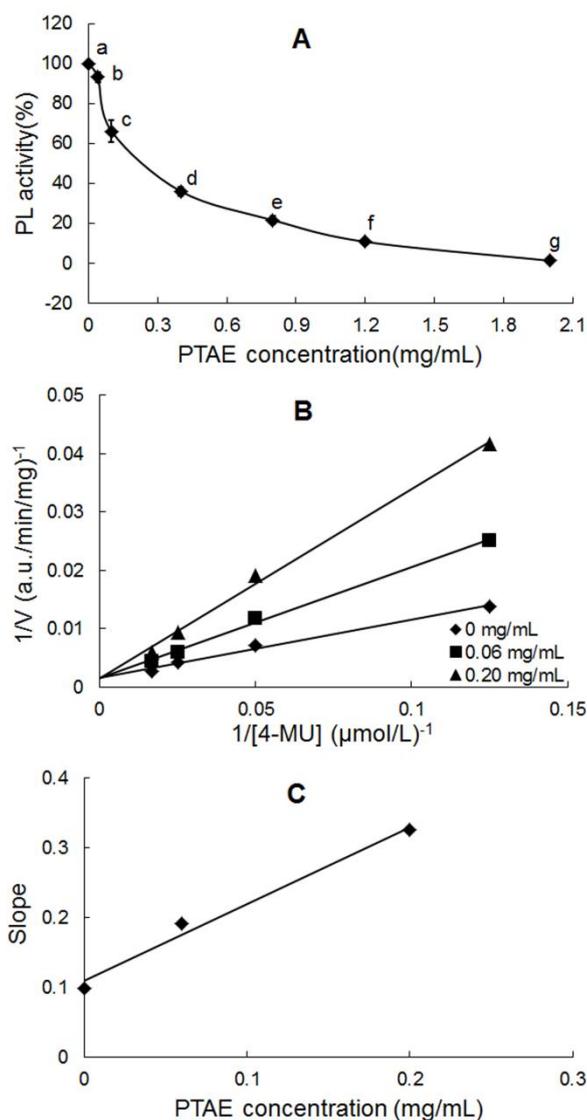


Figure 4. Dose-response effect of PTAE on PL activity *in vitro* and Characterization of the inhibition. (A) A PL solution (1 mg/mL) was incubated with a 0.1 mM 4-MU oleate solution and treated for 30 min with PTAE (final concentrations: 0, 0.04, 0.10, 0.40, 0.80, 1.20 and 2.00 mg/mL). The amount of 4-MU produced by lipase was measured with an F-2500 spectrofluorometer at an excitation wavelength of 362 nm and an emission wavelength of 448 nm. The percent change caused by PTAE was expressed relative to the measurement of the 0 mg/mL PTAE-treated group, which was defined as 100%. Each value is the mean of triplicate experiments; (B) Lineweaver-Burk plot of the inhibition of PL activity by PTAE. (C) K_i determination for PTAE. Slopes from the Lineweaver-Burk plots (B) were plotted against the concentration of PTAE.

solubility and was easily obtained from pu-erh tea in contrast with the aforementioned inhibitors.

HMGCR, the cellular enzyme required for cholesterol biosynthesis, plays a central role in the production of cholesterol in the liver, which produces 50% or more of the total cholesterol in the body.³⁹ High cholesterol levels have

been associated with CVD.⁴⁰ It is well known that the commonly used drugs, statins, can lower cholesterol levels by inhibiting HMGCR.⁴¹ However, statins also have some side effects, including muscle pain, an increased risk of diabetes and abnormalities in liver enzyme tests.^{42, 43} Animal experiment has shown that pu-erh tea significantly lowers LDL-C and TG levels, and elevates HDL-C level.⁶ In contrast with statins, pu-erh tea is consumed as a natural daily drink and has no adverse effects.⁴⁴⁻⁴⁶ In this study, PTAE, extracted from pu-erh ripe tea, competitively inhibits HMGCR for the NADPH binding site with an IC_{50} of 0.39 mg/mL and K_i of 0.33 mg/mL. We believe that additional benefits of PTAE will be revealed for preventing high cholesterol, heart disease or CVD.

LCAT is a plasma enzyme secreted exclusively by the liver into the plasma that catalyzes the synthesis of cholesteryl esters in human plasma, which is a key step in the reverse cholesterol transport pathway.⁴⁷⁻⁵⁰ LCAT also plays a critical role in the maturation and remodeling of HDL. Genetic disorders involving this enzyme are primary causes of HDL deficiency, which is a metabolic disorder characterized by low plasma levels of HDL-C in strong association with an increased risk of CVD.⁵¹⁻⁵⁴ It has been identified that pu-erh tea suppresses hyperlipidemia, lowers the risk of CVD, increases serum levels of HDL-C and reduces LDL-C.⁷ This study demonstrates that PTAE has negligible effects at concentrations <0.76 mg/mL but that it significantly increase LCAT activity at concentrations >0.76 mg/mL. This result suggests that one of pathways of the mediation of blood lipid metabolism by pu-erh tea involves an increase in LACT activity, especially through increasing the serum level of HDL-C.

PL is a key enzyme involved in the efficient digestion of dietary TGs. Excess TGs can cause the formation of a large deposit of adipose tissue, thereby contributing to obesity.^{21, 55, 56} Recently, much research has focused on the screening of hundreds of species of medicinal plants for potential lipase inhibitory activity, and tea is one kind of target medicinal plants to be screened. Compared with green tea and oolong tea, pu-erh tea has lower total tea catechins content, but it is able to decrease the serum TGs level more efficiently.⁴ This study shows that PTAE dose-dependently inhibits PL activity *in vitro* with an IC_{50} of 0.20 mg/mL and that PTAE acts as a competitive inhibitor for PL with K_i of 0.10 mg/mL. It is also demonstrated that the chemical compounds in PTAE include total catechins (12.512%), gallic acid (43.38%), caffeine (4.86%), amino acids (3.12%) and soluble sugar (10.55%). Both pu-erh tea extract and gallic acid have been indicated to inhibit PL activity in dose-dependent manners *in vitro* and the inhibitory activity of gallic acid is approximately 11 times higher than that of pu-erh tea extract.³⁴ It has been demonstrated that a mixture of catechins that are high in EGCG and ECG dose-dependently inhibit PL activity and that when they are purified, only those with a galloyl moiety can inhibit its activity.⁵⁷ Therefore, we speculate that the PL-inhibitory activity of PTAE is mainly attributed to gallic acid and that its remaining activity can be attributed to the other components, especially synergistic effect of EGCG and ECG.

Experimental

Materials and chemicals

Pu-erh ripe tea 7572 was kindly donated by Menghai Tea Factory (Yunnan Province, China). Standard compounds of C, EC, EGC, ECG, EGCG, gallic acid, caffeine, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), and NADPH were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), heparin sodium, 4-MU oleate and 4-MU were purchased from Aladdin Company (Shanghai Province, China). A PAF Acetylhydrolase Assay Kit and an LCAT kit were obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and Niujiemei Biotechnology (Shanghai Province, China), respectively. The other chemicals used were obtained from Kelong Biotechnology Company (Sichuan Province, China).

Ethics statement

An ordinary housing facility was used in this study in accordance with the national standard "Laboratory Animal-Requirements of Environment and Housing Facilities" (GB 14925-2010). All experiments were approved by the Institutional Animal Care and Use Committee of Southwest University. Male rats (*Rattus norvegicus*) and rabbit (*Chinese white rabbit*) were obtained from Tengxin Biotechnology Company (Chongqing, China) and were housed individually under standard laboratory conditions. The temperature was maintained at approximately 24±1 °C using a ventilation fan and forced air heating. The animals were raised for 4 d and they would adapt themselves to the new environment for 3 d, then we would begin our experiments on the fourth day. They were fasted for 24 h before they were killed, and water was provided daily ad libitum.

Preparation and detection of PTAE

Pu-erh tea (Sample 7542, 18-30 mesh) was extracted with distilled water (1:20, w/v) at 100 °C for 30 min. After filtration, the extract was eluted (solvent A, water; and solvent B, ethanol) using a gradient program. The target product, termed PTAE, was freeze-

Table 2. Gradient elution system for detection of catechins, gallic acid and caffeine in PTAE.

Time (min)	A (%)	B (%)
	95	5
5	95	5
10	90	10
15	90	10
35	68	32
45	68	32
46	95	5

Catechins, gallic acid and caffeine were identified by HPLC under the following conditions: Hypersil BDS C18 column (250 mm×4.6 mm, 5 μm); UV detection at 278 nm; mobile phase A, 1% acetic acid; mobile phase B, acetonitrile; flow rate, 0.9 mL/min; temperature, 35 °C; and injection volume, 20 μL.

dried and stored at -80 °C until further use.

Catechins, gallic acid and caffeine were identified by HPLC (Shimadzu Corporation, Kyoto, Japan), according to the previous method with slight modifications.⁵⁸ The chromatographic conditions were as follows: Hypersil BDS C18 column (250 mm×4.6 mm, 5 μm); UV detection at 278 nm; mobile phase A, 1% acetic acid; mobile phase B, acetonitrile; flow rate, 0.9 mL/min; temperature, 35 °C, and injection volume, 10 μL. The gradient elution program is shown in Table 1.

Amino acids were estimated spectrophotometrically by the ninhydrin assay at a wavelength of 570 nm.⁵⁹ Soluble sugar was determined at 620 nm using the anthrone colorimetric method.⁶⁰ Total TFs, TRs and TBs were measured as previously described.⁶¹

Preparation of crude enzyme extract

Three male rats weighing between 220-250 g each were used as sources of crude enzyme in this study. Crude enzyme extract was prepared in accordance with the previous method with slight modifications.^{62, 63} During the experimental period, the rats were fasted for 24 h and were killed the following morning. They were rapidly decapitated with scissors, and then aliquots of each liver (approximately 1.5 g) were immediately weighed and placed into 4:1 (v/w) cold homogenization medium (0-4 °C) at pH 7.4. The homogenization medium contained 0.01 M potassium phosphate, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 10 mM sodium EDTA, and 50 mM sodium chloride. The livers were cut into small pieces with scissors and homogenized twice at 4 °C with an XFD H-Speed Dispersator (NingBo Scientz Biotechnology Company, China) for 18 s at 20,000 g. Each homogenate was centrifuged at 12,000 g for 30 min at 4 °C to remove cell debris and mitochondria. Then, the supernatant fraction was centrifuged at 12,000 g for 30 min at 4 °C, and the resulting supernatant fraction was frozen immediately and stored at -80 °C. The supernatant fraction, namely the crude enzyme extract, was later used for analysis of HMGR activity.

A male white rabbit weighing 2.2 kg was fasted for 24 h prior to the start of the experiment. The rabbit was placed under ether anesthesia and its carotid artery was cut, then the blood was removed and placed into a beaker containing of 2 mL heparin sodium solution. A blood thinner was used for preventing clots. The blood (approximately 50-60 mL) was centrifuged at 3,000 g for 30 min at 4 °C, and the supernatant fraction to be used for analysis of LCAT activity was removed, transferred to centrifuge tubes and immediately stored as crude enzyme extract at -80 °C.

Lp-PLA₂ activity

Lp-PLA₂ activity was measured with a commercially available kit based on the previous methods.^{64, 65} Briefly, standard human plasma Lp-PLA₂ and PTAE (final concentrations: 0, 0.05, 0.10, 0.20, 0.40, 0.80 and 1.60 mg/mL) were divided into aliquots, transferred to 96-well microliter plates, and mixed with a substrate solution containing 2-thio PAF (86.96 μM) and assay buffer.^{17, 66} The reaction was allowed to proceed at room

temperature for 20 min, and then 10 μL 5,5'-Dithio bis-(2-nitrobenzoic acid (DTNB) was added to terminate the reaction. Enzyme activity (U) was equal to an absorbance change per minute of 0.01 at 405 nm (Model 680 Microplate Reader, Bio-Rad Laboratories, Inc., US) and was expressed as U/min/mL of the standard human plasma Lp-PLA₂. Kinetic analyses were performed in the presence of varying concentrations of PTAE (0.30, 0.60 and 0.80 mg/mL) across a range of 2-thio PAF levels (86.96, 173.92 and 260.88 μM) to evaluate kinetic properties.

HMGR activity

HMGR activity was analyzed as previously described with slight modifications.^{39, 63, 67} First, crude enzyme extract was incubated at 37 °C for 30 min. The preincubation mixture for measuring HMGR activity contained the following concentrations of components in a total volume of 1.65 mL: 75 μL HMG-CoA (465 μM), 1 mL of 0.01 M potassium phosphate (pH 7.4), 150 μL crude enzyme extract, and 250 μL PTAE (final concentrations: 0, 0.08, 0.30, 0.45, 0.60, 0.70 and 0.90 mg/mL). The tubes were incubated at 37 °C for 15 min, and then 175 μL NADPH (2.65 mM) was added to the preincubation mixture to initiate the reaction. A total of 0.5 mL of the mixture was transferred to new tubes and diluted in 4 mL distilled water, and then absorbances were determined with a UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 340 nm. Following incubation for 60 min at 37 °C, 0.5 mL of the mixture was removed and re-measured in the same manner. The activity was expressed as the uptake of NADPH $\mu\text{mol}/\text{min}/\text{mL}$ crude enzyme extract. To ascertain the mode of inhibition by PTAE and obtain an accurate K_i , we assayed HMGR activity at a variety of different PTAE concentrations (0, 0.20 and 0.40 mg/mL) in the presence of increasing concentrations of NADPH (81.20, 162.40, 243.60 and 324.80 μM).

LCAT activity

LCAT activity was measured with a commercially available kit. Esterase activity was determined by the hydrolysis of a water-soluble substrate, *p*-nitrophenyl butyrate (PNPB), as previously described.^{68, 69} The reaction mixture contained 50 μL crude enzyme extract, 10 μL PTAE (final concentrations: 0, 0.01, 0.02, 0.76, 1.14 and 1.52 mg/mL), 100 μL reaction liquid, 100 μL PNPB and 790 μL buffer. Following incubation of the mixture for 20 min at 37 °C, the formation of *p*-nitrophenoxide was monitored with a UV-VIS spectrophotometer at 400 nm. The activity was expressed as the synthesized *p*-nitrophenoxide $\mu\text{mol}/\text{min}/\text{mL}$ crude enzyme extract.

PL activity

PL activity was measured according to the previous method using 4-MU oleate as a substrate with slight modifications.²⁰ A total of 250 μL PTAE (final concentrations: 0, 0.04, 0.10, 0.40, 0.80, 1.20 and 2.00 mg/mL) and 500 μL of 0.1 mM 4-MU oleate solution, which was dissolved in a buffer consisting of 13 mM Tris-HCl, 150 mM NaCl, and 1.3 mM CaCl₂ (pH 8.0), were mixed completely in a tube. Then, 500 μL lipase solution (1 mg/mL) was added to the aforementioned buffer to begin the

enzymatic reaction. A total of 1 mL of 0.1 M sodium citrate (pH 4.2) was added to terminate the reaction following incubation at 25 °C for 30 min. The amount of 4-MU produced by lipase was measured with an F-2500 spectrofluorometer (Hitachi Limited, Kyoto, Japan) at an excitation wavelength of 362 nm and an emission wavelength of 448 nm. The activity was expressed as a.u./min/mg protein. To further investigate the nature of this interaction, the kinetics of increasing concentrations of 4-MU oleate (8.00, 20.00, 40.00 and 60.00 μM) were examined alone and in the presence of 0, 0.30, 1.00 and 2.00 mg/mL PTAE.

Statistical analysis

Data were reported as the mean \pm SD of triplicate experiments. All figures were created with Microsoft Office Excel 2010 and Adobe Photoshop 7.0, and all data were analyzed using IBM SPSS Statistics 19. Differences between means were determined using two-tailed tests, and a $P < 0.05$ was considered statistically significant.

Conclusions

Pu-erh tea and its components are believed to be effective on blood lipid metabolism in animal experiments. Pu-erh tea as blood lipid metabolism regulator has many advantages such as aqueous solubility, easy accessibility and daily consumption. In this study, we determine that PTAE could mediate the metabolism of blood lipids by controlling the activities of key enzymes, for example, by inhibiting Lp-PLA₂, HMGR and PL activities and elevating LCAT activity. PTAE is a significant component of pu-erh tea; therefore, we speculate that PTAE is partly responsible for the positive effects of this tea on regulating blood lipid metabolism and that it may be an alternative hypolipidemic drug. Animal experiment would be done to confirm the effects of PTAE on blood lipid metabolism enzymes *in vivo* in the subsequent experiments. Further work is needed to search for the specially characterize synergistic and individual effects of phytochemical components that account for its ability to regulate blood lipid metabolism.

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