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In Vitro Inhibitory Effect of Triterpenoidal Saponins from Platycodi Radix on Pancreatic Lipase

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In the process of investigating anti-obesity effect of Platycodi Radix, we found that aqueous extract of Platycodi Radix might inhibit intestinal absorption of dietary fat by inhibiting pancreatic lipase (PL) activity. In order to clarify the anti-obesity mechanism of Platycodi Radix, activity-guided isolation was performed to find active components. The total saponin fraction of Platycodi Radix appeared to have a potent inhibitory activity against the hydrolysis of triolein emulsified with phosphatidycholine by pancreatic lipase *in vitro*. Based on these results, further purification of active components yielded 10 known triterpenoidal saponins, among these compounds, platycodin A, C, D, and deapioplatycodin D exhibited significant inhibitory effects on PL at the concentration of 500 μg/mL with 3.3, 5.2, 34.8, and 11.67% pancreatic lipase activity vs control, respectively. Platycodin D was found to inhibit the PL activity in a dose-dependent manner. Therefore, the anti-obesity effect of Platycodi Radix might be due to the inhibition of pancreatic lipase by its saponins.

Key words: Platycodi Radix, Anti-obesity, Pancreatic lipase, Platycodins

INTRODUCTION

Obesity is one of the increasing health problems in most developed countries and its prevalence is also increasing in developing countries. It is considered to be a risk factor associated with the genesis or development of major chronic diseases, including cardiovascular disease, diabetes, and cancer (Bray, 2002; Flegal *et al.*, 2002). It is rapidly becoming a worldwide epidemic, with significant consequences in terms of clinical burden and economic costs in treating its complications, therefore new effective approaches are needed. Currently, the strategy for preventing and/or treating obesity includes: (i) the suppression of dietary intake, (ii) increasing thermogenesis, and (iii) altering metabolism (Bray, 2000). The inhibition of dietary fat absorption has been reported to be one of the most effective ways of managing obesity. Pancreatic lipase (PL) has recently been in the limelight as a target for management of obesity. The drug orlistat (Xenical), a hydrogenated derivative of lipostatin derived from *Steptomyces toxitricini*, is a typical PL inhibitor that interferes with digestion of triglyceride and thereby reduces absorption of dietary fat. Clinical trials (Drent *et al.*, 1995a and 1995b) supported the contention that inhibiting lipase could lead to significant reduction in body weight in some patients. Therefore, orlistat has been developed as a promising agent for obesity therapy. In addition, the existence of lipase inhibitors in various plant species has been investigated and reported, including *Cassia nomame* (Hatano *et al.*, 1997; Yamamoto *et al.*, 2000), *Camelia sinensis* (Han *et al.*, 2001), *Salacia reticulata* (Yoshikawa *et al.*, 2002), *Dioscorea nipponica* (Kwon *et al.*, 2003) and grape seed (Diego *et al.*, 2003).

Platycodi Radix, the roots of *Platycodon grandiflorum* (Jacq.) A.DC. (Campanulaceae), has been traditionally used as an antiphlogistic, antitussivic and expectrantic agent in China, Korea, and Japan. Although modern pharmacology researches suggest that extracts from Platycodi Radix possess wide-ranging health benefits, presently Platycodi Radix still is a hot research topic in South Korea, Japan, because of its potential uses in

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health care. Recently, it has been reported that Platycodi Radix appeared to prevent hypercholesterolemia and hyperlipidemia (Kim *et al.*, 1995). In China and Korea, the fresh roots of *P. grandiflorum* are eaten as pickles for preventing obesity. However, the mechanism of anti-obesity and anti-hyperlipidemic effects of Platycodi Radix still remains to be clarified. Previously, we found that an aqueous extract of Platycodi Radix might inhibit intestinal absorption of dietary fat by inhibiting hydrolysis of the fat by PL (Han *et al.*, 2000). Further research showed that the anti-obesity effect of Platycodi Radix in mice fed with high fat diet might be due to the inhibition of intestinal absorption of dietary fat by crude saponins (Han *et al.*, 2002). As a result of our continued study of anti-obesity effects of Platycodi Radix, we isolated platycodins from methanol extract of Platycodi Radix and examined their inhibitory effect on PL activity *in vitro*. In present paper, we describe the inhibitory effect of platycodins on PL.

MATERIALS AND METHODS

Instrument and reagents

UV spectrum was measured with a Backman Du-7500 UV-VIS Spectrophotometer and FT-IR spectrum was recorded on a Jasco FT/IR-5300 spectrometer on KBr. NMR spectra were measured using a Varian Unity 500 NMR Spectrometer and ESI-Ion trap/MS was performed on a Finngan MAT LCQ electrospray multiple-stage tandem mass spectrometer. Column chromatography was performed using silica-gel (silica gel H 60 for TLC use, Qingdao Ocean Chemical Industry Co., Qingdao, China), Sephadex (LH-20, Amersham Biosciences, Sweden), Lichroprep RP-18 (ODS, 40-63 μm, Merck, Germany) and Macroreticular resin (ZTC-1, Tianjin Zheng Tian Cheng Purification Technology Co., Tianjin, China). Thin layer chromatography (TLC) on pre-coated silica-gel 60 F_{254} (0.25mm, Merck, Germany) and RP-18 F_{254S} plates (0.25mm, Merck, Germany). Triolein and pancreatic lipase (EC 3.1.1.3) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), other chemicals were of analytical grade. Orlistat (Xenical®, Roche Pharmaceuticals, Basel, Switzerland) capsule material was extracted with ethanol to make a nominally 1 mg/mL stock solution.

Plant materials

Platycodi Radix, the roots of *Platycodon grandiflorum* (Jacq.) A.DC., was purchased from Guangfu Road Market of Changchun, China and identified by Prof. Li Xiang Gao at College of Chinese Material Medicine, Jilin Agricultural University.

Preparation of aqueous extract, total saponin and inulin from Platycodi Radix

Aqueous extract was prepared as follows: Dried Platycodi Radix 1 kg were extracted with boiling water under reflux for 3 h, the aqueous solution was concentrated to give a dark brown extract.

The crushed dried Platycodi Radix (4.5 kg) were extracted with methanol five times under reflux for 6 h, yielding 465 g of a brown solid extract, 400 g of which was suspended in 2 L MeOH and precipitated by adding 5 fold volume acetone. The supernatant was concentrated *in vacuo* to yield brown gum, the resulting gum was suspended in 1 L H_2O and defatted with Et_2O. The aqueous layer was extracted with aqueous saturated *n*-BuOH. The resulting *n*-BuOH solution was concentrated *in vacuo* to yield the *n*-BuOH fraction (97 g). The *n*-BuOH fraction (90 g) was suspended in 1 L water, the soluble fraction was subjected to a macroreticular resin column chromatography and eluted firstly using water for desugarizing and decoloring, then eluted using EtOH-water gradients, combined various eluted fractions together and concentrated *in vacuo* till without EtOH odor, the concentrated solution was freeze-dried to yield total saponins powder (61.9 g).

After methanol extraction, the residues of Platycodi Radix were extracted with warm water for 2 h, the aqueous extract was precipitated by adding 5 fold volume ethanol, the precipitated pellets were suspended in 1 L water, then were freeze-dried to yield white powder (inulin fraction).

Isolation of platycodins from total saponin

The above-mentioned total saponin lyophilized powder (50 g) was chromatographed on a silica gel column and eluted by using a $CHCl_3$-MeOH-H_2O (65:35:10, v/v/v, lower layer) elution system to yield 10 fractions, which were further separated by repetitive silica gel, Sephadex LH-20, ODS column chromatography. Ten known triterpenoidal saponins were isolated. The structures of these compounds were identified by comparing physicochemical and spectroscopic data with previous reported results (Tada *et al.*, 1975; Ishii *et al.*, 1978, 1984; Xu *et al.*, 1999, 2000).

Measurement of PL activity

Lipase activity was determined by measuring the rate of release of oleic acid from triolein. Enzyme activity was expressed as nmol oleic acid release/ (L reaction mixture·h). A suspension of triolein (80 mg), phosphatidylcholine (10 mg), lecithin (10 mg), and taurocholic acid (5 mg) in 9 mL of 0.1 M *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.0) containing 0.1 M NaCl was sonicated for 5 min. This sonicated substrate suspension (0.1 mL) was incubated with 0.05 mL (10 U) of pancreatic lipase and 0.1 mL of various concentration of sample solutions for 30 min at 37 °C in a final volume of

0.25 mL. The amount of release of oleic acid produced was determined based on the method described by Zapf (Zapf *et al.*, 1981) with a minor modification (Tsujitu and Okuda, 1983). The incubation mixtures were added to 3 mL aliquots of a 1:1 (v/v) mixture of chloroform and *n*-heptane containing 2% (v/v) methanol and extracted by shaking the tubes horizontally for 10 min in a shaker. The mixture was centrifuged at 2,000 g for 10 min, and the upper aqueous phase was removed by suction. Copper reagent (1 mL) was then added to the lower organic phase. The tube was shaken for 10 min, the mixture was centrifuged at 2,000 g for 10 min, and 0.5 mL of the upper organic phase, which contained copper salts of the extracted free fatty acids (FFA), was treated with 0.5 mL of 0.1% (v/v) bathocuproine in chloroform containing 0.05% (w/v) 3-(2)-*tert*-butyl-4-hydroxy-anisol. The absorbance was then measured at 480 nm. In addition, PL activity was expressed as μmol oleic acid released per mL reaction mixture per hour.

Statistical analysis

The results are expressed as means ± standard errors (S.E.). The data were statistically analyzed by one way ANOVA. Fishers Protected LSD and/or Scheffes tests were used to determine the significance of differences among the groups. Differences with $P<0.05$ were considered significant.

RESULTS

Effects of various fractions from Platycodi Radix on PL activity in *vitro*

In the previous experiments (Han *et al.*, 2000), we found that an aqueous extract of Platycodi Radix might inhibit intestinal absorption of dietary fat by inhibiting PL activity. In order to clarify the anti-obesity mechanism of Platycodi Radix, as a continued study of anti-obesity effects of Platycodi Radix, activity-guided isolation was performed to seek active fractions and active components. After solvent fractionation, the inhibiting effects of various fractions on PL activity were compared, the aqueous extract of Platycodi Radix was found to inhibit the PL activity in a dose-dependent manner in the assay system using triolein emulsified with phosphatidylcholine. While inulin, which is known to be a major component of Platycodi Radix, did not inhibit PL activity *in vitro* (Table I). Total saponins were found to have significant inhibiting activity on PL, while ethyl ether fraction, *n*-butyl alcohol fraction have no inhibiting activity on PL *in vitro* (Table I).

Table I. Effects of fractions from Platycodi Radix on PL activity *in vitro*.

Sample	Sample final concentration μg/mL	Activity of pancreatic lipase 100% vs. control
Control		100
Total saponins	500	41.7± 3.7
n-BuOH extract	500	87.6±17.8
Et_2O extract	500	110.4±12.7
Inulin	500	89.4± 1.8
Orlistat*	500	10.2± 0.5

* Positive control

Effect of total saponin fraction on PL activity *in vitro*

Above-mentioned results suggested that anti-obesity actions of the aqueous extract of Platycodi Radix might be attributed in part to its total saponin fractions. To clarify the active substances of Platycodi Radix, we examined the effects of the major secondary metabolites from Platycodi Radix, total saponin fractions, on PL activity. As previous report (Han *et al.*, 2000), the total saponin fraction strongly inhibited the PL activity in the assay system using triolein emulsified with phospatidylcholine.

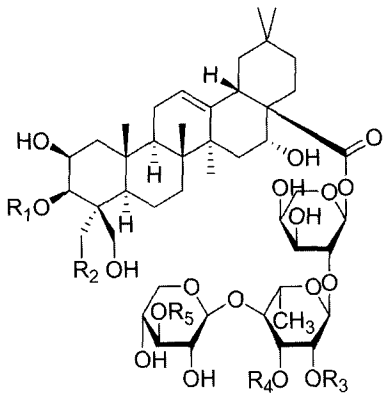
Identification of platycodins and their effects of on PL activity

Activity-guided isolation of total saponin fractions was furnished 10 compounds. The structures (Fig. 1) of these compounds were identified as platycodin V, platycodin A, C, D, platycodin D_2, D_3, deapioplatycodin D, D_3, polygalacin D, and polygalacin XI by comparing physicochemical and spectroscopic data with literatures (m.p., IR, MS and NMR) (Tada *et al.*, 1975; Ishii *et al.*, 1978; Ishii *et al.*, 1984) and our previous reported results (Xu *et al.*, 1999; Xu *et al.*, 2000).

Furthermore, we examined inhibitory activity of these compounds on PL (Table II). platycodin A, C, D and deapioplatycodin D exhibited significant inhibitory effect on PL at the concentration of 500 μg/mL, with 3.3%, 5.2%, 34.8%, and 11.67% pancreatic lipase activity vs. control, respectively.

Platycodin D inhibits PL activity *in vitro*

Platycodin D, as a major pharmaceutically active component of Platycodi Radix, whose several pharmaceutical activities have been investigated (Mita *et al.*, 1979; Arai *et al.*, 1997; Kim *et al.*, 2001; Choi *et al.*, 2002; Shin *et al.*, 2002) at single compound level. In addition platycodin D was frequently used as marker substance, which was reviewed in our latest article (Xu *et al.*, 2004), for quality control and evaluation of Platycodi Radix. It is due relatively higher content of platycodin D among total saponins and relatively easier isolation from Platycodi Radix than other individual saponins. Therefore, platycodin D might be potential candidate compound for new medicine development among all individual platycodins.



Compounds	R_1	R_2	R_3	R_4	R_5
Platycodin D	Glucosyl	OH	H	H	Apiosyl
Platycodin A	Glucosyl	OH	Ac	H	Apiosyl
Platycodin C	Glucosyl	OH	H	Ac	Apiosyl
Deapioplatycodin D	Glucosyl	OH	H	H	H
Platycodin D_2	Laminaribiosyl	OH	H	H	Apiosyl
Platycodin D_3	Gentiobiosyl	OH	H	H	Apiosyl
Deapioplatycodin D_3	Gentiobiosyl	OH	H	H	H
Platycodin V	Laminaribiosyl	OH	Ac	H	Apiosyl
Polygalacin D_2	Laminaribiosyl	H	H	H	Apiosyl
Polygalacin XI	Laminaribiosyl	H	Ac	H	Apiosyl

Fig. 1. Compounds isolated from Platycodi Radix

Table II. Effects of platycodins on PL activity *in vitro*

Sample	Sample final concentration μg/mL	Activity of pancreatic lipase 100% vs. control
Control		100
Total saponins	500	41.7 ±3.7*
Platycodin D	500	34.8 ±12.4*
Platycodin V	500	70.7 ±2.82
Platycodin D_2	500	94.5 ±6.3
Platycodin D_3	500	100.8 ±2.85
Deapioplatycodin D	500	11.67±6.75*
Deapioplatycodin D_3	500	64.4 ±0.25
Polygalacin XI	500	108.0 ±9.64
Polygalacin D_2	500	109.3 ±3.5
Platycodin A	500	3.3 ±1.7*
Platycodin C	500	5.2 ±0.1*
Orlistat	500	10.2 ±0.5*

* P<0.05

In the presence of platycodin D, the PL activity was inhibited in a concentration-dependent manner (Table III). The IC_{50} value of platycodin D was calculated to be 450 μg/mL.

Table III. Effects of platycodin D on PL activity *in vitro*

Platycodin D μg/mL	Lipase activity 100% vs. control
0	100 ± 1.0[a]
125	109.8 ± 1.1[a]
250	95.8 ± 1.6[a]
500	34.5 ±11.4[b]
1000	18.3 ± 0.9[b]

Results are Mean ± S.E., n=3. Mean not sharing a letter differ, p<0.05.

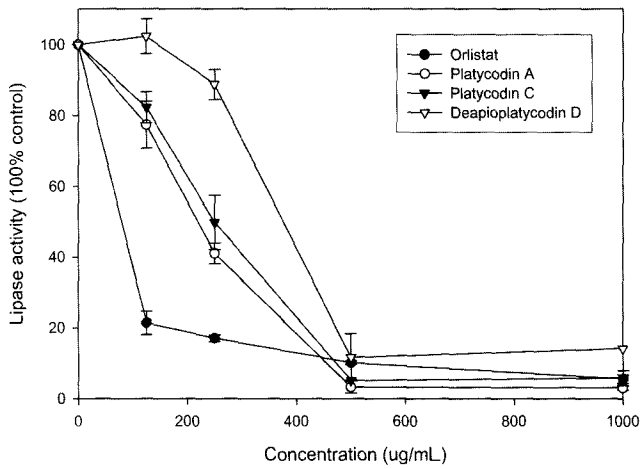


Fig. 2. Effects of platycodin A, C, and deapioplatycodin D on PL activity *in vitro*.

Platycodin A, C, and deapioplatycodin D inhibits PL activity *in vitro*

Platycodin A, C, and deapioplatycodin D showed strong inhibitory activities against PL, which almost approached positive medicine Orlistat at the concentration of 500 μg/mL (Table II). As a result, concentration-dependent effects of platycodin A, C, and deapioplatycodin D against PL were further investigated. It was found that platycodin A and C exhibited a dose-dependent manner in our present assay system by using triolein emulsified with phosphatidylcholine, while deapioplatycodin D did not exhibit a dose-dependent effect (Fig. 2).

DISCUSSION

Obesity results from an imbalance between energy intake and energy expenditure. Fat tissue hydrolysis and release fatty acid as well as triolein by lipase; on the other hand, fatty acids origin from food or fatty acids which were released in decomposed metabolism of fat tissue by pancreatic lipase. Anti-obesity mechanism of drug lie in decomposing unnecessary fat tissue and inhibiting absorption of fat from food, among them, two key enzymes are lipase and pancreatic lipase. Exciting lipase or

inhibiting pancreatic lipase can act anti-obesity effect. The major form of dietary fat is triglyceride or neutral lipid. A triglyceride molecule cannot be directly absorbed across the intestinal mucosa. Rather, it must be first digested into one 2-monoglyceride and two free fatty acids. The enzyme that performs this hydrolysis is pancreatic lipase, which is delivered into the lumen of the gut as a constituent of pancreatic juice.

As previously reported (Han *et al.*, 2000), the oral administration of aqueous extract of Platycodi Radix for 8 weeks prevented obesity and the associated increases in parametrial adipose tissue weight and hepatic triacylglycerols caused by consumption of a high-fat diet containing 40% beef tallow. Verger (Verger *et al.*, 1984) reported that dietary fat was hydrolyzed during the course of digestion by pancreatic lipase. The two main products formed by the hydrolysis of pancreatic lipase are fatty acids and 2-monoacylglycerols (Hernell *et al.*, 1990), these lipolytic products are mixed with bile salts, dispersed as micelles, and carried in this form to the site of fat absorption. Lipid absorption takes place in the apical part of the plasma membrane of epithelial cells or enterocytes lining the gut. In the present study, we first attempted to examine the effects of the various fractions from Platycodi Radix on pancreatic lipase activity. Total saponins were found to have a significant inhibiting activity on PL. Activity investigation was further done on each single saponin compound isolated from total saponins. Platycodin A, C, D, and deapioplatycodin D exhibited significant inhibitory effects on PL at the concentration of 500 μg/mL (Table II), platycodin D was found to inhibit the PL activity in a dose-dependence manner in the assay system using triolein emulsified with phosphatidylcholine (Table III).

It was reported that various saponins isolated from foodstuffs or natural medicine have antiobesity (Kawano-Takahashi *et al.*, 1986; Han *et al.*, 2001) or anti-hypolipidemic (Kimura *et al.*, 1983) actions. In present study, we found that the total saponins as well as single compound from Platycodi Radix, such as platycodin A, C, D, and deapioplatycodin D, strongly inhibited PL activity (Table II, Fig. 2). Therefore, it seems likely that the antiobesity and hypolipidemic actions of Platycodi Radix may be attributed in part to its single compound. The results indicated that these triterpenes compounds have the potential to be obesity-preventive agents. It has been reported that the peak-area ratios of platycodin A, C, D, of commercial sample from China and Korea appeared at 1:2:3, and 2:4:1 based upon their HPLC patterns, respectively, while the Japanese botanical garden or wild types samples appeared at 1:2:1 (Saeki *et al.*, 1999). Considering relative abundance as well as PL inhibitory activity, platycodin A, C, and D seem to have been major compound excerting lipase inhibitory action in Platycodi Radix. In addition, we found that relative low molecular weight saponin compounds, such as platycodin D, deapio-platycodin D, platycodin A, C possess relative higher inhibiting activity than those of relative high molecular weight saponin compounds (the latter possess one or two more glucose in structures than earlier, Fig. 1), such as platycodin V, D_2, D_3; meanwhile, pologalacin XI and platycodin V with similar molecular weight (with different prosaponins), as well as, polygalacin-D_2 and platycodin-D_2 with similar molecular weight (with different prosaponin) possess weak or no inhibiting activity. Structure-activity relationships of active components remain to be further studied to clarify their functional group and active configuration. More studies and *in vivo* experiments are also required to ascertain whether platycodins may provide effective natural anti-obesity agents.

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