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Cholesterol-lowering effect of platycodin D in hypercholesterolemic ICR mice

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Abstract

This study investigates the in vivo hypocholesterolemic action of platycodin D and its in vitro evidence for the cholesterol-lowering properties. In order to examine the effects of platycodin D on hypercholesterolemia in male ICR mice, platycodin D with doses of 15, 30 or 50 mg/kg was orally administered for 8 weeks. Changes in body weight and daily food intake were measured regularly during the experimental period. Final contents of triglyceride and different types of cholesterol in the serum, livers and feces were determined. The effects of platycodin D on cholesterol metabolism were further investigated with several in vitro assays, including antioxidant effect on low density lipoprotein oxidation, inhibition of human acyl-coenzyme A:cholesterol acyltransferase (hACAT) and serum lipoprotein associated-phospholipase A_2 (Lp-PLA_2), as well as the regulation of farnesoid X receptor. The formation of insoluble complex between platycodin D and cholesterol was also investigated. Following an eight week experimental period, the body weights of platycodin D-fed mice were less than those of control mice on a high cholesterol diet by 11.2±5% ($P<0.01$) with 15 mg/kg platycodin D, 11.7±5% ($P<0.01$) with 30 mg/kg platycodin D, and 23.4±7.9% ($P<0.0001$) with 50 mg/kg platycodin D, respectively. A decrease in daily food consumption was also noted in most of the treated animals. Triglyceride and cholesterol concentrations were decreased in serums and livers, but increased in feces. Some of the in vitro observations revealed that the hypocholesterolemic effect of platycodin D is partly associated with inhibition to hACAT activity and antagonism to the farnesoid X receptor as well as the formation of insoluble complex with between platycodin D and cholesterol. Both in vivo and in vitro results demonstrate a potential value of platycodin D as a novel cholesterol-lowering and anti-atherogenic candidate.

Keywords: Platycodin D; Cholesterol lowering; Anti-obesity; ACAT inhibition; Farnesoid X receptor

1. Introduction

Over the past three decades, the prevalence of obesity, non-insulin-dependent diabetes, dyslipidemia, hypertension, and cardiovascular disease has risen to reach epidemic proportions. Reduction in the concentration of blood lipids, especially cholesterol, is a major goal in several primary and secondary prevention initiatives. A variety of drugs and potent lead compounds have been developed (Harris et al., 1997; Harwood et al., 1993; Morehouse et al., 1999; Oakenfull and Topping, 1983).

Recently, various saponin-containing natural products have emerged as potential lead compounds, drug alternatives or nutritional supplements. In these products, saponins function as active ingredients effective in controlling of hypercholesterolemia, hyperlipidemia, hyperglycemia, and obesity (Bramlett et al., 2003; Han et al., 2002).

Platycodi Radix is the root of *Platycodon grandiflorum* A.De (Campanulaceae) (Ishii et al., 1981, 1984). It is widely used in

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traditional Chinese medicine as an expectorant for pulmonary diseases and a remedy for respiratory disorders. Platycodin saponins are the primary bioactive components of Platycodi Radix (Ishii et al., 1981) responsible for a diversity of effects including anti-inflammatory and anti-allergic activities, inhibition of tumor growth, augmentation of immune response, and stimulation of skin cell apoptosis. (Choi et al., 2001a,b; Kim et al., 2001; Ahn et al., 2005). In the last decade, platycodin saponins have generated a renewed interest due to its novel pharmacological potentials for treating diseases of adulthood such as hyperlipidemia, hypertension, diabetes, and obesity (Han et al., 2000, 2002).

We have already observed that platycodin saponins exert anti-obesity and hypolipidemic effects (Zhao et al., 2005). During the experiment, we noticed the serum and hepatic cholesterol levels were decreased as well. Since the former experiments were designed on purpose to investigate the hypolipidemic effects, many of the crucial factors involved in the cholesterol homeostasis and atherogenesis were not clarified. Therefore, further investigations were needed to manifest the cholesterol lowering and anti-atherogenic potency based on the in vivo and in vitro evidences.

In the present study, platycodin D, the major saponin of Platycodi Radix was used instead of mixture of saponins. Hypercholesterolemic ICR mice were also used for in vivo testing of various doses of platycodin D during a 12-week study, and then a series of in vitro investigations were carried out to explore if those cholesterol lowering effects of platycodin D are correlative to the atherogenic risk factors such as LDL oxidation (Havel et al., 1955; Yancey and Jerome, 1998), human acyl-coenzyme A:cholesterol acyltransferase (hACAT) inhibition, lipoprotein associated-phospholipase A_2 (Lp-PLA_2) inhibition (Hakkinen et al., 1999; Macphee, 2001), and farnesoid X receptor activation (Bramlett et al., 2000).

2. Materials and methods

2.1. Preparation of platycodin D

The raw sample (100 kg) of Platycodin Radix was extracted with methanol and partitioned sequentially with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The *n*-butanol fraction was then subjected to Diaion HP-20 resin (Mitsubishi Chemical Corporation, Japan), and the fractions eluted at 60–80% of methanol were collected to obtain 90 g of crude saponins. The crude saponins were further purified by repeated silica gel (Merck, Germany) chromatography to obtain the purified platycodin D. The process was repeated several times until a sufficient quantity of platycodin D was obtained. The purified platycodin D was identified on the basis of R_f, FAB-MS (=1225.38), and [^{13}C]-NMR spectra compared with the authentic platycodin D, generously provided by Dr. Eun Bang Lee, Professor Emeritus at Seoul National University (Fig. 1). The purity was assessed based on the HPLC chromatogram of ZORBAX SB-Aq ODS C_{18} (Agilent, Palo Alto, CA) equipped with the evaporative laser scattering detector (Sedex 75, Sedere, France). The other saponins, such as polygalacin D, platycodin A, and platycodin D_3, were obtained as the by-products of platycodin D separation as previously described (Zhao et al., 2005).

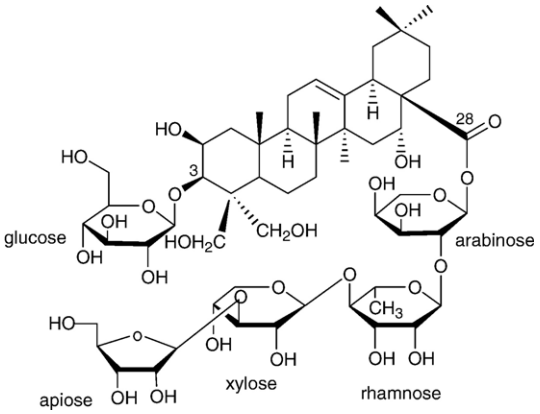

Fig. 1. Structure of platycodin D. Platycodin D is a triterpenoid bidesmoside, composed of an aglycone moiety, 3-Glc and 28-*O*-Api-Xyl-Rha-Ara.

2.2. Oral administration of platycodin D to hypercholesterolemic ICR mice

The high cholesterol (Vlahcevic et al., 1990) diet contains 1% cholesterol and 0.2% cholic acid (Sigma-Aldrich, St. Louis, MO) in a granulated commercial chow (Shin Chon Feed Co. Ltd, Incheon, Korea). The high cholesterol-free diet consisted of the granulated commercial chows. Both chows were prepared by the same manufacturing processes. Both of the processed chows contain 19% protein, 61% carbohydrate, 7% fat, and 10% water.

Male ICR mice (6 weeks) were obtained from the Animal Center of Seoul National University. The mice (six in each cage) were housed at a controlled temperature (21–23 °C) and ambient humidity (50–60%) with a 12 h light–dark cycle. The animals were provided with unrestricted access to the normal diet chow and water for one week prior to any experiments.

After the animals had been fed with high cholesterol diet for one week, only those with over 200 mg/dl of blood cholesterol were selected for use in the experiment. Twenty-four selected mice were randomly divided into four groups (six animals in each group), three platycodin D test groups and one high cholesterol control group. To the three test groups, 15, 30 or 50 mg of platycodin D per kg of animal body weight was orally administered once per day for 8 weeks. To the high cholesterol control group, 0.9% saline was served instead of platycodin D as a negative control. All of the above animals were provided with the HC diet throughout the animal test. A normal group (n=6) fed with a high cholesterol-free diet was used for comparison and was supplied with the same volume of 0.9% saline instead of platycodin D.

All of the animal procedures were carried out in accordance with the “Policy and Regulation for the Care and Use of Laboratory Animals” in Seoul National University.

2.3. Body weight and food intake

The body weight and daily food intake were recorded twice per week. The food intake efficiency in each group was evaluated twice per week by monitoring the food consumption (g) in each cage for two consecutive days, and was calculated on per animal per day basis.

2.4. Collection of the serum, liver and fecal specimens

At the end of the experimental period, the animals were kept on food supply for 2 more h after platycodin D administration and then fasted for 14 h. Blood samples were collected by periorbital blooding and kept at room temperature for 2 h, followed by centrifugation at 3500 ×*g* for 10 min to obtain serum. The serum was stored at −81 °C until analysis. After blooding, the animals were sacrificed by cervical dislocation. The livers were excised, weighed, and stored at −81 °C for further analysis. The fecal outputs of about 40–70 mg for each specimen, were collected 48 h in advance of the end of the experimental period. The collected fecal specimens were freeze-dried and weighed.

2.5. Determination of serum parameters

The activities of serum alanine amino-transferase (ALT) and aspartate amino-transferase (AST), the concentrations of serum triglyceride and total cholesterol, were all determined using commercially available kits (Eiken Chemicals Co. Ltd., Tokyo, Japan). They are colorimetric assays depending on detection of a high-colored end-product at 490–520 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

The concentration of high density lipoprotein-cholesterol was determined with a high density lipoprotein-cholesterol diagnostic reagent (Kyowamedex Co. Ltd, Japan) using a Hitachi-747 auto-analyzer (Hitachi Co., Tokyo, Japan). The concentration of low density lipoprotein-cholesterol was detected with Roche 2nd generation low density lipoprotein-cholesterol plus reagent using a Hitachi-7170 auto-analyzer (Hitachi Co., Tokyo, Japan).

2.6. Lipid extraction and classification

Hepatic and fecal lipids were extracted essentially as previous described (Folch et al., 1951). In brief, the samples were extracted with chloroform and methanol (2:1, 1 ml for 1 mg sample), followed by sonication for 5 min. The tubes were centrifuged at 3000 ×*g* for 10 min to remove the insoluble material. Then the distilled water was added to the supernatant, and the lower phase was collected and dried under nitrogen. The dried extract was then dissolved in 100 µl of chloroform and classified by aminopropyl-bonded solid phase extraction column (Alltech, Deerfield, IL). Aminopropyl columns (500 mg) were placed in the vacuum manifold (Alltech, Deerfield, IL) and washed twice under vacuum (~10kPa) with 4 ml portions of *n*-hexane. The lipid extracts in 100 µl of chloroform were applied to the column under vacuum and the chloroform was pulled through. Then, the column was eluted with 6 ml of chloroform: 2-propanol (15:1, v/v) to elute neutral lipids. The eluate containing neutral lipids was dried under nitrogen and subjected to HPLC.

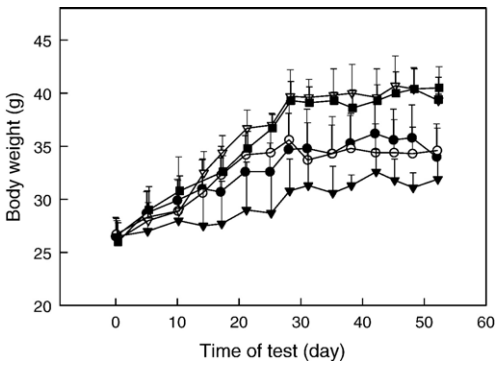

Fig. 2. Body weight changes ($n=6$),. Platycodin D was administered with doses (*p.o.*) of 0 (▽), 15 (●), 30(○) and 50 (▼) mg/kg, respectively; the high cholesterol diet was fed throughout the test. A normal group (high cholesterol-free control, (■) received no platycodin D treatment. The data are expressed as mean±S.D. and are considered to be significantly different at $P<0.05$ by the unpaired Student's *t*-test. $P<0.05$–0.001 (from the 5th week of test) in 15 and 30 mg/kg platycodin D groups; $P<0.05$–0.01 (from 4th week) and $P<0.001$–0.0001 (from 5th week of test) in 50 mg/kg platycodin D group.

Table 1
Food intake efficiency during platycodin D administration ($n=6$)

Week	1	2	3	7	8
Control	100±4.2	100±5.9	100±4.5	100±1	100±16.0
15 mg/kg PD[d]	90.5±2.7[a]	88.4±6.2	80.0±2.0[a]	70.6±10.9[a]	81.9±4.6
30 mg/kg PD[d]	54.4±0.2[b]	74.0±12.2[a]	68.2±1.4[b]	80.4±3.0[a]	80.0±4.0
50 mg/kg PD[d]	42.0±1.2[c]	65.4±5.8[b]	53.1±3.2[b]	65.0±3.0[b]	67.9±2.0[a]
Normal (HC-free)[d]	89.6±13.9	85.1±2.8[a]	82.1±4.0[b]	93.2±18.8	82.5±16.8

The data are mean±S.D. and significantly different at [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ by the unpaired Student's *t*-test.
[d]The results are expressed as % of the high cholesterol control.
PD, platycodin D., HC, high cholesterol.

2.7. Determination of neutral lipids

For quantitative analysis of lipid profile, the extracted lipids were analyzed with HPLC equipped with a LiChrosphere 100 Diol column (5 µm, 4×250 mm, Merck), a Hitachi pumps (L-6200), an evaporated light-scattering detector (Sedere, Sedex75, Vitry-sur-Seine, France), and an auto injector (Shimadzu, SIL-9A). An adequate eluent system was used with A: hexane–acetic acid (99:1. v/v) and B: hexane–isopropanol–acetic acid (84:15:1, v/v/v). The running conditions are as follows: 0–25 min (12–100% B), 25–26 min (100% B), 26–29 min (100–12% B), and then equilibrated with 12% B for 10 min with a flow rate of 1 ml/min, the column temperature of 50 °C, the detector temperature of 50 °C and the N_2 pressure of 2 bar.

2.8. hACAT inhibition

In vitro inhibitory activity against ACAT-1 or ACAT-2 was determined according to the method described by Cho et al. (2003a) using the expressed human ACAT-1 or ACAT-2 from the Hi5 cell as the enzyme source. The microsomal fraction was prepared by centrifugation using a Beckman L8-M ultracentrifuge (Palo Alto, CA) and SW55.1 rotor.

2.9. Farnesoid X receptor binding activity

The co-activator recruitment assay for farnesoid X receptor activation was carried out with recombinant farnesoid X

Table 2
Serum parameters after 8 weeks' platycodin D administration ($n=6$)

Parameter	15 mg/kg PD	30 mg/kg PD	50 mg/kg PD	Control	Normal
Diet	HC	HC	HC	HC	HC-free
TG (mg/100 ml)	96.1±29.4[b]	94.5±41.0[a]	116.8±20.7[a]	144.6±32.5	110.0±20.8[a]
TC (mg/100 ml)	95.0±13.0[b]	112.9±8.1[b]	121.1±24	152.8±35.2	116.3±12.5[a]
HDL-C (mg/100 ml)	48.4±7.9	66.0±22.8	61.8±24.8	62.2±6.1	74.9±8.6
LDL-C (mg/100 ml)	40.8±20.0[a]	40.1±28.5[a]	31.0±9.6[a]	70.6±28.3	60.8±44.8
HDL-C /LDL-C[d]	1.6±0.6	3.9±2.7[a]	2.1±0.2[b]	1.0±0.4	2.2±1.0[c]
TC/HDL-C[d]	2.1±0.4	1.9±0.7	1.9±0.3[a]	2.6±0.5	1.5±0.2[c]

The data are mean±S.D. and significantly different at [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ by the unpaired Student's t-test.
[d]The discrete parameters of each measurement were used in statistical analysis after being counted and averaged.
PD, platycodin D., HC, high cholesterol., TG, triglyceride., TC., total cholesterol.
HDL-C, high density lipoprotein cholesterol.
LDL-C, low density lipoprotein cholesterol.

receptor protein and its natural ligand, chenodeoxycholic acid (CDCA). Farnesoid X receptor activation was detected by addition of 1 nM of DELFIA-Eu-N1-labelled anti-His antibody (Perkin-Elmer, Foster City, CA) using an optical microplate reader (Wallac Victor 2, Perkin-Elmer) as previously described (Cho et al., 2003b). The platycodin D fraction was dissolved in 50% ethanol and various platycodin D fractions were prepared in 3 μl volume for the assay in a total reaction volume of 43 μl.

2.10. Complex formation between platycodin D and cholesterol in vitro

The standard solution of cholesterol (3 mg/ml ≈ 7.75 mM) of the cholesterol assay kit (Eiken Chemicals, Japan) was used as it stands. An aliquot of 100 μl cholesterol solution was added to the same volume of platycodin D solutions with a series of concentrations. The mixed solutions were incubated at room temperature with mild shaking for 3 h, then, the cholesterol concentrations in the suspension were determined using the same assay kits as described above, after removal of precipitation by centrifugation. The triglyceride standard solution (3 mg/ml) was used for comparison. The complexation abilities of other platycodin saponin compounds, such as polygalacin D, platycodin A, and platycodin D_3 were also investigated.

2.11. Data analysis

Values were expressed as means±standard deviations (S.D.). Differences in mean values between groups were analyzed by a one-way analysis of variance and the unpaired Student's t-test. Statistical significance was considered to exist at $P<0.05$.

3. Results

3.1. Changes in body weight and daily food intake

The oral administration of platycodin D to hypercholesterolemic ICR mice for eight weeks resulted in a significant reduction in the body-weight gain in a dose-dependent manner (Fig. 2). Doses of 15, 30 and 50 mg/kg of platycodin D in the three test animals yielded reductions in body weight gain of 11.2±5% ($P<0.01$), 11.7±5% ($P<0.01$) and 23.4±7.9% ($P<0.0001$), respectively, as compared to the high cholesterol control. The high cholesterol diet alone yielded no difference in body weight gain when compared to the high cholesterol-free group, indicating that the supplementation of cholesterol itself has no appreciable effect on body-weight gain.

A similar decline was seen in the daily food consumption at platycodin D treatment (Table 1). In contrast to a previous report (Han et al., 2002), our observations demonstrated a significant

Table 3
Hepatic parameters after 8 weeks' platycodin D administration ($n=6$)

Parameter	15 mg/kg PD	30 mg/kg PD	50 mg/kg PD	Control	Normal
Diet	HC	HC	HC	HC	HC-free
Liver mass (g)	1.47±0.38[a]	1.83±0.14	1.81±0.23	1.98±0.19	1.84±0.16
TG (mg/g liver mass)[d]	12.19±2.19	15.84±0.30	6.01±3.02[a]	19.67±9.14	15.80±4.41
CE (mg/g liver mass)[d]	14.36±2.01[b]	11.08±5.89[b]	14.19±3.27[b]	27.71±7.84	6.80±2.62
FC (mg/g liver mass)[d]	6.26±0.87	5.36±0.99	6.74±2.41	6.74±0.70	6.85±2.05
CE/FC[e]	2.31±0.29[b]	2.09±1.05[a]	2.28±0.84[b]	4.06±0.76	0.97±0.11
ALT (KU/l) (serum)[f]	19.8±0.5[a]	19.1±7.1	22.0±1.2[a]	25.1±1.5	20.4±2.7[a]
AST (KU/l) (serum)[f]	42.6±1.5[c]	42.3±3.4[b]	57.6±3.2[a]	79.2±3.2	57.0±2.7

The data are mean±S.D. and significantly different at [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ by the unpaired Student's t-test.
[d]TG (triglyceride), CE (cholesterol Ester), FC (free cholesterol) were all quantified by the above mentioned HPLC chromatography compared to the standard curve of TG, CE and FC. [e]CE/FC is the mean±S.D. of each counted ratio. [f]KU (Karmen unit).
PD, platycodin D., HC, high cholesterol.

Table 4
Fecal parameters after 8 week's platycodin D administration

Parameter (mg/100 mg feces)	15 mg/kg PD (HC diet)	30 mg/kg PD (HC diet)	50 mg/kg PD (HC diet)	Control (HC diet)	Normal (HC-free)
Total lipid extract[b]	12.2±1.9	12.0±1.3[a]	14.6±3.0[a]	9.9±1.8	6.3±2.1[a]
TG	7.3±3.0[a]	5.1±1.4	4.8±1.5	4.1±0.7	3.1±1.2
Cholesterol[c]	1.3±0.2	1.8±1.0	3.4±1.2[a]	1.5±0.4	1.1±0.7

The data are mean±S.D. and significantly different at [a]$P<0.05$ by the unpaired Student's *t*-test.
[b]Total lipid extract was obtained by Soxhlet lipid extraction.
[c]Cholesterol was determined by commercial kits and includes cholesterol ester, free cholesterol and some cholesterol metabolites which remained as an un-oxidized 3-hydroxyl residues.
PD, platycodin D., HC, high cholesterol., TG, triglyceride.

decrease in food consumption in a dose-dependent manner. This tendency was most marked in the first two weeks of the test. From that point on, it steadily regressed toward the control value until the end of the test period except in the 50 mg/kg platycodin D group, where the food intake remained somewhat lower than in the control group throughout the experiment.

3.2. Biochemical analysis of the serum

As shown in Table 2, the high cholesterol diet group obtained an elevated serum total cholesterol, low density lipoprotein cholesterol and triglyceride, but a decreased high density lipoprotein, suggesting an effective induction of hypercholesterolemia by supplementation of cholesterol in diet was effectively established in ICR mice. platycodin D administration in doses of 15, 30 and 50 mg/kg lowered the elevated cholesterol levels to 65.5%, 73.7% and 79.6% of high cholesterol control, respectively. Furthermore, the concentrations of low density lipoprotein cholesterol declined to 57.8%, 56.8% and 43.9% of high cholesterol control, respectively. The concentrations of high density lipoprotein cholesterol in most of the platycodin D-treated animals, however, were not significantly altered. Table 2 also shows that platycodin D

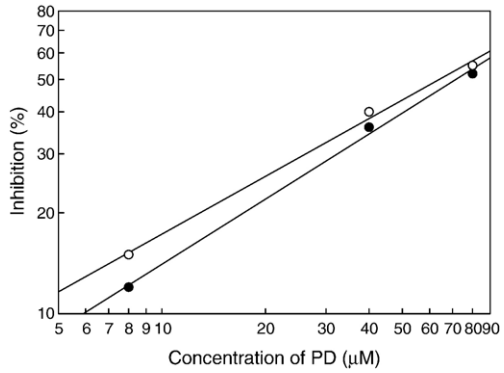

Fig. 3. Inhibition of ACAT activities by platycodin D. A series of concentrations of platycodin D was added to the assay systems of ACAT-1 (●), ACAT-2 (○). The inhibition (%) was expressed as a logarithm scale type. Inhibitory activity = [(activity without platycodin D − activity with platycodin D)/activity without platycodin D] × 100.

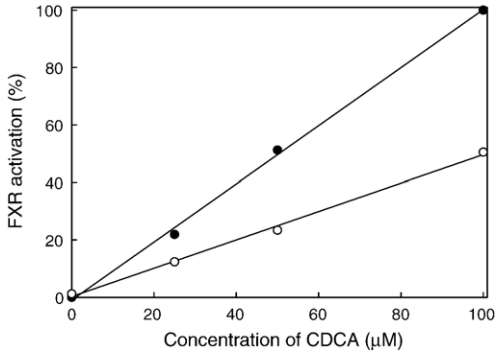

Fig. 4. Antagonizing effect of platycodin D to farnesoid X receptor activity. The farnesoid X receptor (FXR) was activated by CDCA (●) and antagonized by 40 nM platycodin D (○). The antagonizing effect of platycodin D was expressed as a decreasing% of fluorescence intensity by CDCA + platycodin D against CDCA activation.

administration lowered the elevated triglyceride concentrations to 65.4–81.0% of high cholesterol control.

3.3. Biochemical analysis of the liver

The concentrations of hepatic triglyceride were decreased by 43–68% in the groups treated with different doses of platycodin D (Table 3). The change of hepatic cholesterol contents was mainly noted in its ester form (cholesterol ester), which has shown a unanimous decrease in all test groups by 48–60%. In contrast, free cholesterol concentration was almost unaltered in either test or high cholesterol control groups. Taken together, the value of cholesterol ester/free cholesterol in high cholesterol control has enhanced to 4.06 (the high cholesterol-free control is 0.97±0.11), while the platycodin D treatment manifestly lowered the elevated ratio of cholesterol ester/free cholesterol to 2.06–2.31. On the other hand, no abnormality in the activities of serum ALT and AST was observed; for example of AST, the values slightly increased in high cholesterol control compared to high cholesterol free group, but AST activities were distinctly regressed by 26–27% in doses of 15 and 30 mg/kg of platycodin D tested animals. Not surprisingly, the liver weights were

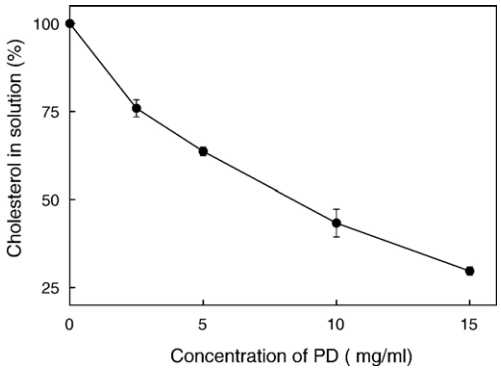

Fig. 5. Formation of complex between cholesterol and platycodin D. The original concentrations of cholesterol were prepared as 3 mg/ml. The platycodin D was added in a series of concentration and shaken at room temperature for 3 h. Then, the concentration of cholesterol in the solution was determined after removal of precipitation by centrifugation. Data are means of triplicate determinations.

slightly decreased from 1.98±0.19 g of high cholesterol control group to 1.47±0.38 ($P<0.05$), 1.83±0.14, and 1.81±0.23, respectively, responsive to 15, 30, and 50 mg/kg platycodin D.

3.4. Biochemical analysis of feces

In contrast to the blood and hepatic lipid profiles, the fecal total lipid, triglyceride and total cholesterol showed a certain degree of increase instead, respectively; i.e. 21–47% increase in total lipid output, 17–78% increase in triglyceride, and 0–126% increase in total cholesterol (Table 4). More specifically, the total cholesterol was steadily increased in a dose-dependent manner.

3.5. Effect of platycodin D on atherogenic factors in vitro

In the in vitro assay for ACAT inhibition, as shown in Fig. 3, platycodin D at 81 μM final concentration showed 42% and 41% inhibitory activity against human ACAT-1 and -2, respectively. These isotypes of ACAT are found to behave differently with respect to many biochemical and biophysical characteristics, such as substrate binding efficiency, membrane topology, and distribution of tissue location. The simultaneous inhibition of ACAT-1 and -2 by platycodin D, therefore, might maximize the therapeutic efficiency of platycodin D for lowering the cholesterol levels.

Platycodin D was also tested to antagonize the nuclear farnesoid X receptor (Fig. 4). Platycodin D caused a decrease in farnesoid X receptor activation stimulated by CDCA, a natural ligand of farnesoid X receptor. Treatment with 40 μM final concentration of platycodin D resulted in 40–50% inhibition of the CDCA control value.

On the other hand, platycodin D was proved ineffective as an antioxidant against copper-mediated LDL oxidation (data not shown). Another in vitro assay for Lp-PLA_2 indicated that platycodin D also showed no noticeable inhibition to activity Lp-PLA_2 either (data not shown).

3.6. Formation of complex between platycodin D and cholesterol

Platycodin D was found to form insoluble complex with cholesterol. Fig. 5 shows that platycodin D concentration at 50% cholesterol precipitation is 8.3 mg/ml (≈6.77 mM), showing an approximate 1:1 ratio between platycodin D and cholesterol in stoichiometry. In comparison, platycodin D did not form complex with triglyceride. It was also noted that the formation of the complex is related to the polarity of platycodin saponins. For instance, polygalacin D and platycodin A that have a lower polarity than platycodin D, showed a stronger affinity to cholesterol, while platycodin D_3, another platycodin saponins with a higher polarity showed little complexion (data not shown).

4. Discussion

Platycodi Radix has been traditionally used as food and used in folk remedies and some of its pharmacological actions have been partly confirmed by modern science (Kim et al., 2001; Choi et al., 2001a,b; Ahn et al., 2005). Recently, Platycodi Radix and its major components, the platycodin saponins, have been promoted as supplements and medicinal alternatives for many adulthood diseases, but experimental evidences have been relatively few and sometimes discrepant.

The data presented in the present paper are consistent with the hypolipidemic effects previously reported for platycodin saponins (Zhao et al., 2005). In cholesterol-fed mice, the administration of platycodin D caused a distinct decrease in the serum triglyceride, total cholesterol and low density lipoprotein cholesterol, and accordingly a certain amount of decrease in the ratio of total cholesterol/high density lipoprotein cholesterol. The reduction of low density lipoprotein cholesterol was more pronounced than that for any other type of cholesterol. Since the concentration of high density lipoprotein cholesterol was not much changed, the cholesterol-lowering effect is in fact exclusively attributed to the low density lipoprotein cholesterol decline, making the most characteristic change involved in cholesterol modulation by platycodin D. Similar results had been previously reported in some of other synthetic saponins, such as tiqueside and pamaqueside. Those saponins demonstrated a similar plasma cholesterol-lowering effect occurred exclusively in the non-high density lipoprotein fractions, (Heck et al., 2000; Morehouse et al., 1999; Oakenfull and Topping, 1983; Uusitupa, 1999) as a consequence of the inhibition of cholesterol absorption.

Some evidence does exist in the literature (Harwood et al., 1993; Morehouse et al., 1999) for the formation of physical insoluble complex with cholesterol, by which saponins are postulated to inhibit cholesterol absorption (Heck et al., 2000; Morehouse et al., 1999; Oakenfull and Topping, 1983; Uusitupa, 1999). Digitonin and tomatine have shown approximate 1:1 complex formation (Harwood et al., 1993), while tiqueside was found to form adducts containing multiple cholesterol molecules per saponin with approximate stoichiometry of 1:5. To our in vitro observation (Fig. 5), the incubation of platycodin D and cholesterol solutions caused precipitation with the approximate stoichiometry of 1:1. This reaction is influenced by the polarity of platycodin saponins, i.e. those with low polarity showed stronger complexion ability (data not shown). The actions of platycodin D on cholesterol absorption and excretion are still obscure at a molecular level, but so far as our in vitro investigations manifested, it is likely that, as a subsequence such complex formation, cholesterol and its metabolites excreted in fecal outputs are remarkably increased up to 226% of high cholesterol control with treatment of platycodin D up to 50 mg/kg (Table 4) and the dietary cholesterol is depleted by precipitation with platycodin D in vivo. If so, the more platycodin D is ingested, the more cholesterol will be depleted as shown in the results.

This physical interaction of complexation is not occurred in triglyceride, however, together with fecal cholesterol augmentation, the triglyceride contents in fecal outputs has also increased upon platycodin D administration, for which we previously reported that platycodin D may be specifically bound to pancreatic lipase and competitively inhibit the enzyme

activity, causing more triglyceride to be excreted without digestion (Han et al., 2002).

We also noticed that the above mentioned nonsystemic actions would be insufficient to cover all cholesterol-lowering effect exerted by platycodin D. Therefore, systemic actions of platycodin D were further evaluated in vitro by determining influences on the atherogenic risk factors (Accad et al., 2000).

ACAT is an allosteric enzyme and plays an important role in the esterification of cholesterol to facilitate intracellular storage and intercellular circulation via incorporation into apolipoprotein-B (apo-B) containing lipoproteins. Numerous reports with in vitro and in vivo experiments have proved that ACAT plays a critical role in the development of atherosclerosis under pathological conditions through foam cell formation (Chang et al., 2000). Recently, the enzyme was found to be present as two isoforms in mammals, ACAT-1 and ACAT-2, with separate genes around 44–47% of low amino acid homology and different tissue distribution as well as a speculated different membrane topology in the endoplasmic reticulum. platycodin D exhibited an inhibitory activity in vitro against recombinant human ACAT-1 and ACAT-2 (Fig. 3), which agrees well with the in vivo result of hepatic cholesterol decrease in ICR mice, especially the decrease in ester form of cholesterol (Table 3), inferring that ACAT inhibition might be one of the crucial pathways involved in cholesterol-lowering effect of platycodin D.

The farnesoid X receptor, a member of the nuclear hormone receptor super-family, was recently demonstrated to function as the bile acid receptor, and its relation with the transcriptional regulation of cholesterol 7α-hydroxylase (CYP7A1) was also demonstrated (Bramlett et al., 2000). Conversion of cholesterol into bile acids in the liver is regulated by the rate-limiting enzyme, CYP7A1. CYP7A1 activity is regulated by feedback repression of the bile acids at the transcriptional level. As they have been well documented previously (Davis et al., 2002), CYP7A1 and ileal bile acid binding protein (i-BABP) are responsible for the synthesis of bile acids (Kanda et al., 1998) and the export of bile salt (Kanda et al., 1998), respectively. Both of them are important factors influencing cholesterol excretion. Theoretically, antagonism to farnesoid X receptor can attenuate the feedback repression of bile acid to farnesoid X receptor and accelerate the rate of cholesterol transfer to bile acid. As shown in Fig. 4, the increased cholesterol excretion observed in mice feces could be in part a reflection of antagonizing effect of platycodin D on the farnesoid X receptor. But we have not observed clear evidence to link platycodin D with another two independent atherogenic risk factors, oxidated LDL and Lp-PLA_2.

The liver weight has been slightly reduced (vs. high cholesterol control) on platycodin D treatment. The activities of ALT and AST have decreased as well, suggesting that platycodin D alleviated liver stress related to hypercholesterolemia and thus is beneficial to prevent from becoming a fatty liver.

On the other hand, food consumption in platycodin D-treated animals showed a remarkable decline in a dose-dependent manner. Food consumption can play a role in cholesterol homeostasis as an independent influential factor. Since the restriction in food intake is also correlative to blood cholesterol reduction, it is quite possible that platycodin D has affected cholesterol homeostasis by a simple but efficient way of food intake suppression. In our previous studies, we already figured out that platycodin D's impact on food intake might be statistically important to body weight change (Zhao et al., 2005) and the actions of the cholesterol lowering presented in this paper would endow more nutritional and therapeutic value with platycodin D.

In conclusion, the present study confirms and extends the understanding of the inhibitory action of platycodin D on pancreatic lipase with in vivo evidences of fecal output augmentation. As for platycodin D involvement in cholesterol metabolism, platycodin D possesses ACAT inhibitory effect, which is attributable to lower the hepatic cholesterol storage. Another potential mechanism is to moderately antagonize the farnesoid X receptor binding together with a nonsystemic complex with cholesterol, by which the cholesterol catabolism, transfer and depletion with feces has been enhanced. Further study may be required that platycodin D represents a good potential as a novel hypocholeserolemic lead compound.

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