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Anti-obesity compounds in green leaves of *Eucommia ulmoides*

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ABSTRACT

The anti-hypertensive effect of Eucommia leaves has been confirmed clinically,[1a,b] and the study of their anti-obesity properties has advanced.[2] However, the compounds involved in their anti-obesity effect have not been fully elucidated. In this Letter, we examined the anti-obesity effect of Eucommia green leaf extract (EGLE) divided into five fractions with high porous polystyrene gel and of the compounds isolated, geniposidic acid, asperuloside and chlorogenic acid, respectively. A metabolic syndrome-like clinical model in mice was generated by feeding a 40% high-fat diet to examine the anti-obesity effects of chronic administration of test substance. After 4 weeks, body weight, white adipose tissue weight, plasma triglyceride levels and total cholesterol levels in the model mice were significantly inhibited by the 30% MeOH fraction (containing much higher levels of asperuloside than the other fractions), and these effects were similar to those of EGLE. Chronic administration of isolated asperuloside in Eucommia leaves suppressed increases in model mouse body weight, white adipose tissue weight, plasma triglyceride levels and free fatty acids levels. These results suggest that asperuloside in Eucommia leaves has important anti-obesity effects.

Obesity, which can lead to metabolic syndrome, has recently gained attention as a factor that dramatically increases the risk of arteriosclerotic disease. According to Japanese diagnostic criteria, obesity and metabolic syndrome are considered conditions requiring prevention and/or treatment due to the high risk of complications, including hyperglycemia, hyperlipemia and hypertension, based on accumulation of visceral fat.[3]

Eucommia ulmoides Oliv. belongs to a single species and genus in the plant family Eucommiaceae. The bark of *Eucommia ulmoides* Oliv. has been used traditionally in analgesics, tonics and hypotensives in China.[4] In a similar manner, this bark is employed in Japan for treatment purposes, and is treated as a medical product by the authorities.

The product 'Tochu-cha' available on the Japanese market is the roasted Eucommia leaf, which has also been used in beverages containing geniposidic acid and asperuloside among the iridoid glucosides and chlorogenic acid, a caffeic acid derivative, as major ingredients[5] (Fig. 1). From Eucommia green leaf powder (EGLP), three new iridoids were isolated, together with 12 known compounds.[6] Namba et al. carried out preliminary pharmacological studies of a water-based extract of Eucommia leaf, reporting that a transient anti-hypertensive effect was due to an agonistic effect on the parasympathetic nervous system.[7] Spontaneously hypertensive rats (SHR) fed Eucommia leaf extract exhibited a dose-dependent anti-hypertensive effect.[1a] Moreover, follow-up studies have confirmed that Eucommia leaf extract decreases systolic blood pressure (SBP) in humans after long-term intake, these anti-hypertensive effects are presumed to be due to the activity of geniposidic acid.[1b]

The anti-hyperlipidemic effect of Eucommia leaf extract has been tested in Male Wistar rats fed high-fat diets (HFD). This extract markedly suppressed increases in total cholesterol, plasma triglyceride levels (TG) and hepatic triglyceride.[8] Recent reports

Abbreviations: BMR, basal metabolic rate; Fr. 1, H_2O fractions; Fr. 2, 30% MeOH fractions; Fr. 3, 50% MeOH fractions; Fr. 4, 80% MeOH fractions; Fr. 5, 100% MeOH fractions; EGLP, Eucommia green leaf powder; EGLE, Eucommia green leaf extract; ELE, Eucommia leaf extract; FDR, fructose-drinking rats; FFA, free fatty acids; HFD, fed high-fat diets; NEFA, nonesterified fatty acid; RQ, respiratory quotients; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; TG, triglyceride; WAT, white adipose tissue.

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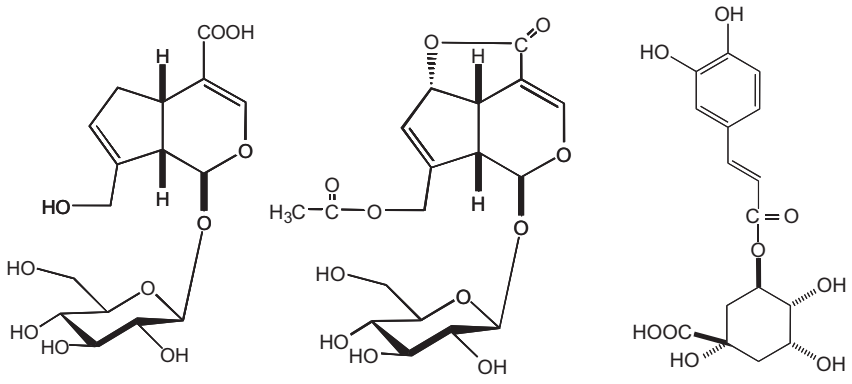

Figure 1. Chemical structures of geniposidic acid, asperuloside and chlorogenic acid.

suggest that Eucommia leaf extract exhibits anti-hyperlipidemic properties by suppressing hepatic fatty acid and cholesterol biosynthesis with simultaneous reduction of plasma and hepatic lipids in high fat-fed hamsters.[9] Experiments with fructose-drinking rats (FDR) fed Eucommia leaf extract (ELE) suggest that long-term ELE treatment effectively prevents the development of insulin resistance and ameliorates abnormal perivascular innervation in FDR.[10]

We reported testing of the anti-obesity effects of ELE and EGLP in female ICR mice fed HFD, both of which markedly suppressed increases in body weight and white adipose tissue (WAT) weight.[11] Moreover, follow-up studies have confirmed that rats fed HFD supplemented with 3% or 9% of ELE and EGLP (HFD-3% or 9% ELE and HFD-3% or 9% EGLP) for a long period of time exhibited significant, dose-dependent decreases in body weight compared to an HFD-Cont. group.[2] These findings suggest that same compounds might participate in anti-obesity effect of Eucommia leaves. However, the compounds involved in their anti-obesity effect have not been fully elucidated. In view of these findings, we examined whether the anti-obesity effects of the principal compounds of Eucommia leaves depend on suppression of increase in body weight and WAT weight.

In this experiments, the anti-obesity effects of EGLE[6,12] divided into the five fractions[12] were evaluated in a metabolic syndrome-like clinical model in mice fed a 40% high-fat diet (HFD).[13] As shown in Table 1, concentrations of geniposidic acid, asperuloside and chlorogenic acid in EGLE and five fractions were measured by HPLC.[14] Test materials[15] were prepared by adding 10% EGLE and fractions (Fr. 1: 7.97%, Fr. 2: 0.76%, Fr. 3: 0.38%, Fr. 4: 0.14%, Fr. 5: 0.004%) in accordance with the weight ratio in EGLP. When the HFD group and the EGLE group were compared, it was found that increase in body weight[16] was significantly inhibited (p <0.01)[17] in mice fed EGLE for 4 weeks (Table 2). Mice fed HFD supplemented with Fr. 1, Fr. 2 and Fr. 3 had significantly decreased (p <0.01) body weight compared to those in the HFD group, while there were no differences in the Fr. 4- and Fr. 5-fed groups. The body weight gain of mice fed HDF supplemented with Fr. 2 and Fr. 3 was modest. EGLE and the five fractions did not affect the food intake of HFD-fed mice.

When the HFD group and the EGLE group were compared, it was found that increase in WAT weight was significantly inhibited (p <0.01) in mice fed EGLE for 4 weeks (Fig. 2). Mice fed HFD supplemented with Fr. 1, Fr. 2, Fr. 3 and Fr. 4 had significantly decreased (Fr. 1: p <0.05, Fr. 2 to Fr. 4: p <0.01) WAT weight compared to the HFD group (Fig. 2). Although the administration of Fr. 1, Fr. 2, Fr. 3 and Fr. 4 reduced WAT weight, this effect was not significant compared to the EGLE group. A marked decrease in weight of WAT per body weight was observed in the EGLE, Fr. 2 and Fr. 4 groups compared with the HFD group (EGLE and Fr. 4: p <0.05, Fr. 2: p <0.01, Fig. 2). Although the administration of Fr. 1, Fr. 3 and Fr. 5 reduced WAT per body weight, these effects were not significant. Adiponectin was among adipocyte-derived endocrine factors inversely correlated with the weight of visceral fat tissue,[18a] and acts to increase insulin sensitivity,[18b] fatty acid oxidation, as well as energy expenditure and reduces the production of glucose by the liver.[18c] We previously reported that chronic administration of ELE and EGLP significantly and dose-dependently increased plasma adiponectin levels, with clear loss of the visceral fat pad in rats in both conditions. In addition, this result was supported by a real-time PCR study in which chronic administration of ELE and EGLP induced the gene expression of PPARγ and adiponectin in the perirenal WAT of rats under both dietary conditions.[2] Further, this study indicated that EGLP was comprised of geniposidic acid 31.8 mg/g, asperuloside 27.8 mg/g and chlorogenic acid 17.8 mg/g. Accordingly, decrease in white adipose tissue weight may contribute to the regulation of body weight.

Table 1
Content of major components on Eucommia leaf

	Geniposidic acid (mg/g)	Asperuloside (mg/g)	Chlorogenic acid (mg/g)
EGLE	63.0	45.2	44.0
Fr. 1	72.2	N.D.	62.3
Fr. 2	16.9	445.9	32.0
Fr. 3	N.D.	12.0	3.4
Fr. 4	N.D.	N.D.	N.D.
Fr. 5	N.D.	N.D.	N.D.

N.D., not detected.

Table 2
Effects of EGLE and 5 fraction supplementation on body weight, food intake HFD-fed mice

	Initial body weight (g/mice)	Final body weight (g/mice)	Body weight gain (g/mice)	Food intake (g/day/mice)
HFD	28.7 ± 0.24	36.0 ± 0.52	7.39 ± 0.65	5.25 ± 0.15
EGLE	28.3 ± 0.56	30.4 ± 0.63**	2.13 ± 0.40**	4.64 ± 0.18
Fr. 1	28.5 ± 0.46	32.0 ± 0.54**	3.54 ± 0.36**	4.26 ± 0.06
Fr. 2	28.3 ± 0.60	28.7 ± 0.44**	0.38 ± 0.24**	5.24 ± 0.07
Fr. 4	28.3 ± 0.60	28.8 ± 0.49**	0.54 ± 0.27**	5.06 ± 0.08
Fr. 5	28.8 ± 0.50	33.9 ± 0.66	5.06 ± 0.43	4.95 ± 0.09
Fr. 3	28.3 ± 0.85	33.5 ± 0.85	5.23 ± 0.53	4.97 ± 0.09

Each value represent the mean ± S.E. Significantly different from HFD.
** p <0.01 (Tukey t-test).

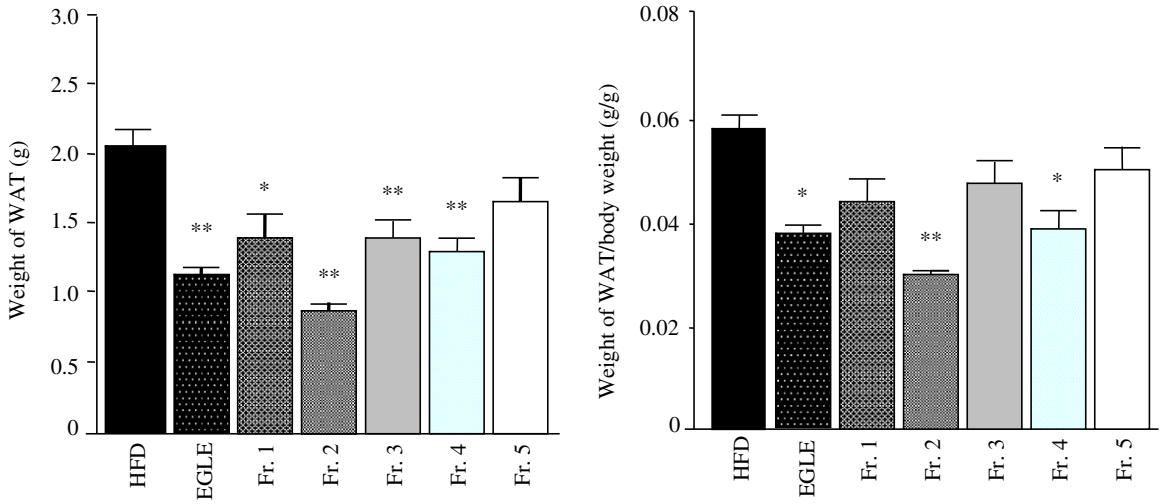

Figure 2. Effects of HFD, EGLE and five fractions on weight of WAT and weight of WAT/body weight in HFD-fed mice. Each value represent the mean ± S.E. Significantly different from HFD, $^{**}p$ <0.01 (Tukey *t*-test). Significantly different from EGLE, not significantly (Tukey *t*-test).

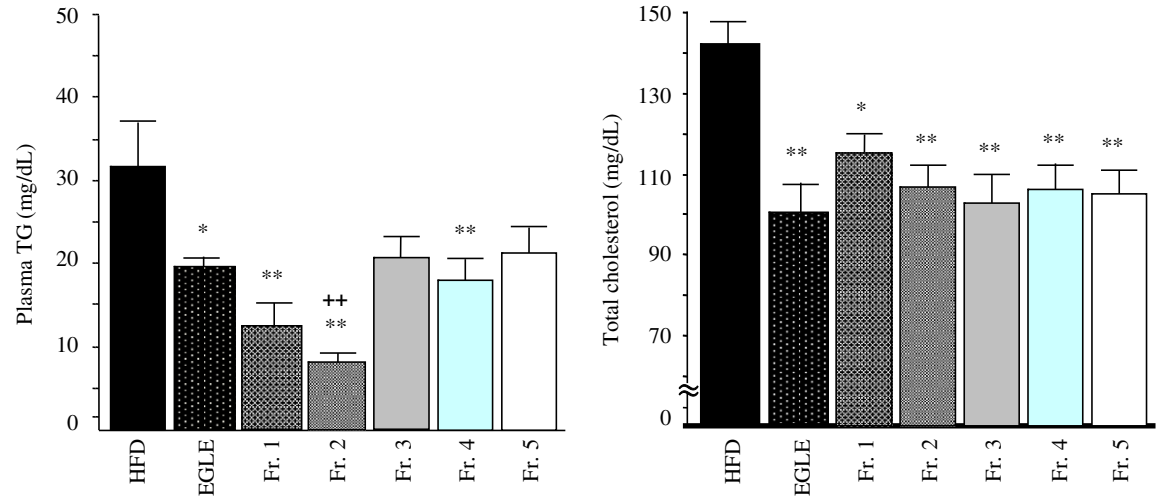

Figure 3. Effects of EGLE and five fractions on plasma TG and total cholesterol in HFD-fed mice. Each value represent the mean ± S.E. Significantly different from HFD, $^{*}p$ <0.05, $^{**}p$ <0.01 (Tukey *t*-test). Significantly different from EGLE, ^{++}p <0.01 (Tukey *t*-test).

Mice fed HFD caused exhibited increase in plasma TG. However, TG was reduced by administration of EGLE, Fr. 1, Fr. 2, and Fr. 4 for 4 weeks (EGLE: p <0.05, Fr. 1, Fr. 2 and Fr. 4: p <0.01, Fig. 3). Administration of Fr. 2 significantly decreased (p <0.05) plasma TG levels compared to the EGLE group. Total cholesterol levels significantly decreased (Fr. 1: p <0.05, EGLP, Fr. 2, Fr. 3, Fr. 4 and Fr. 5: p <0.01) in all groups after 4 weeks compared to the HFD group (Fig. 3). There were no significant differences in total cholesterol level among the EGLE and fraction groups. These results are consistent with a report that the hypolipidemic effects of ELE and EGLP,[11] and rats fed the HFD supplemented with 3% or 9% of ELE and EGLP (HFD-3% or 9% ELE and HFD-3% or 9% EGLP) showed significant dose-dependent decreases in body weight compared to the HFD group.[12]

It has been reported that 35-day administration of Eucommia leaf extract at a high dose (3 g or 6 g dried leaves/kg b.w. of rat/day) or 10-week administration of extract at a low dose (ca. 15.5 mg/day/hamster) did not significantly decrease the body weight of animals.[8,9] Rats administered Eucommia leaf extract at a high dose exhibited a significant decrease in weight of WAT,[8] while Eucommia leaf extract administered at a low concentration to hamsters did not affect WAT weight.[9] These anti-obesity effects and hypolipidemic effects may be affected by differences in component content ratio in Eucommia leaves.

Body weight, WAT weight, plasma TG and total cholesterol in the model mice were significantly inhibited by Fr. 2, and these effects were similar to those of EGLE. These findings suggest that only a limited number of components in EGLE are involved in the anti-obesity effect. Although administration of Fr. 4 reduced WAT weight, plasma TG and total cholesterol, decrease in body weight was not similar to that in the EGLE group. Since the content of Fr. 2 was geniposidic acid (content 16.9 mg/g), asperuloside (content 445.9 mg/g) and chlorogenic acid (content 32.0 mg/g), these findings raise the possibility that the main component with anti-obesity effects was asperuloside.

In the next experiments, the anti-obesity effects of geniposidic acid (isolation from EGLE, purity 98.9%), asperuloside (isolation from EGLE, purity 99.5%) and chlorogenic acid (reagent) were evaluated in a Metabolic syndrome-like clinical model in mice fed HFD.[13,19] Test materials[20] were prepared by adding 10% EGLE and

compounds (geniposidic acid: 0.63%, asperuloside: 0.45%, chlorogenic acid: 0.44%) in accordance with the weight ratio in EGLP. When the HFD group and the EGLE group were compared, it was found that increase of body weight and WAT weight was significantly inhibited (p <0.05) in mice fed EGLE for 4 weeks (Table 3). These results provide further support for the anti-obesity effect observed experiment 1. Mice fed HFD supplemented with asperuloside exhibited significant decrease (p <0.05) in body weight compared to the HFD group, while there were no differences in the geniposidic acid- and chlorogenic acid-fed groups. The body weight gain in mice fed HFD supplemented with asperuloside was inhibited at levels comparable to that with EGLE. EGLE and the three compounds did not affect the food intake of HFD-fed mice.

As shown in Figure 4, mice fed HFD supplemented with EGLE, geniposidic acid and asperuloside for 4 weeks exhibited significant decreases in weight of WAT compared to the HFD group. Mice fed HFD supplemented with asperuloside exhibited significant decrease (p <0.05) in weight of WAT and WAT per body weight compared to the HFD group. Chlorogenic acid did not affect body weight and WAT weight in HFD-fed mice. The HFD-fed mice administered chlorogenic acid at a high dose exhibited significant decreases in body weight, visceral fat mass and plasma leptin and insulin levels compared to the high-fat control group.[21] Based on this finding and the earlier one that ELE and EGLP include different levels of components, it appears that differences in beef tallow content ratio of HFD may affect lipid metabolism. The suppression of increases in model mouse body weight and WAT weight was strongly dependent on asperuloside.

In experiments with the five fractions, total cholesterol levels significantly decreased in all groups, while plasma TG was reduced by administration of EGLE, Fr. 1, Fr. 2 and Fr. 4 after 4 weeks compared to the HFD group. Furthermore, administration of Fr. 2 significantly decreased plasma TG levels compared to the EGLE group. This experiment was performed to examine whether the main components participate in the regulation of plasma TG and free fatty acids (FFA). Administration of EGLE and geniposidic acid, asperuloside and chlorogenic acid for 4 weeks did not significantly alter TG in mice fed HFD, and decrease in TG was observed in the following order: chlorogenic acid > asperuloside > geniposidic acid > EGLE (Fig. 5). Mice fed HFD supplemented with asperuloside and chlorogenic acid exhibited significant decreases (p <0.01) in FFA compared to the HFD group and EGLE group. Real-time PCR analysis showed that chronic administration of ELE and EGLP increased fatty acid β-oxidation in the liver, and in HFD-fed rats, uptake of fatty acids into the liver was increased by ELE and EGLP, followed by an increase in fatty acid β-oxidation and ATP production, which may decrease circulating NEFA levels in HFD-fed rats.[2] In this Letter, administration of ELE and EGLP clearly decreased plasma TG and FFA levels under HFD conditions. The anti-obesity activities of Eucommia leaf in this mouse model may be maintained through secretion and regulation of adipocytokines that depend on the accumulation of visceral fat to improve hyperlipemia. To prove them, chronic administration of 5% EGLE, 0.1% asperuloside and 0.1% chlorogenic acid significantly enhanced the basal metabolic rate (BMR) in rats fed a HFD (Supplementary data). 0.1% asperuloside decreased the respiratory quotients (RQ) more than the HFD control group that might show the acceleration of lipid metabolism, and 0.1% geniposidic acid increased RQ more than the HFD control group that might show the acceleration of glucose metabolism (Supplementary data).

Differences between the two experiments were observed in body weight gain, WAT weight and plasma TG in the 10% EGLE group (Tables 2 and 3, Figs. 2–5). These two experiments were performed under the same conditions. The differences observed may depend on growth processes and differences in the initial body weight of mice. Additional studies are needed to clarify the effect

Table 3
Effects of EGLE and three compounds supplementation on body weight, food intake HFD-fed mice

	Initial body weight (g/mice)	Final body weight (g/mice)	Body weight gain (g/mice)	Food intake (g/day/mice)
HFD	25.9 ± 0.38	35.5 ± 0.95	9.50 ± 0.68	5.51 ± 0.20
EGLE	25.2 ± 0.55	31.7 ± 0.73*	6.53 ± 0.33*	4.08 ± 0.09
Ganiposidic acid	25.4 ± 0.37	33.9 ± 0.61	8.53 ± 0.43	4.93 ± 0.20
Asperuloside	25.5 ± 0.50	31.8 ± 0.89*	6.35 ± 0.49*	4.68 ± 0.20
Chlorogenic acid	25.9 ± 0.44	35.7 ± 1.32	9.83 ± 1.29	4.66 ± 0.36

Each value represents the mean ± S.E. Significantly different from HFD
* p <0.05 (Tukey t-test).

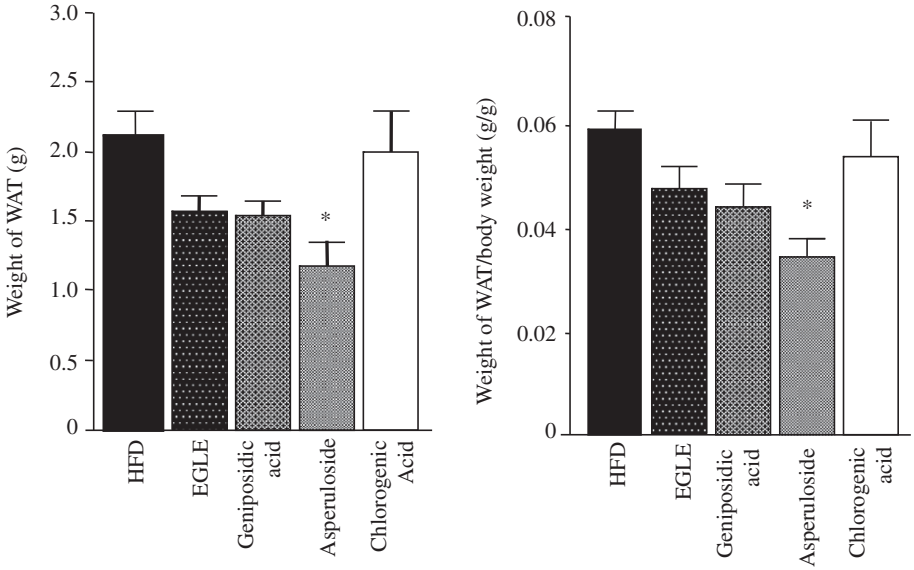

Figure 4. Effects of EGLE, EGLE and three compounds on weight of WAT and weight of WAT/body weight in HFD-fed mice. Each value represent the mean ± S.E. Significantly different from HFD, *p <0.05 (Tukey t-test). Significantly different from EGLE, not significantly (Tukey t-test).

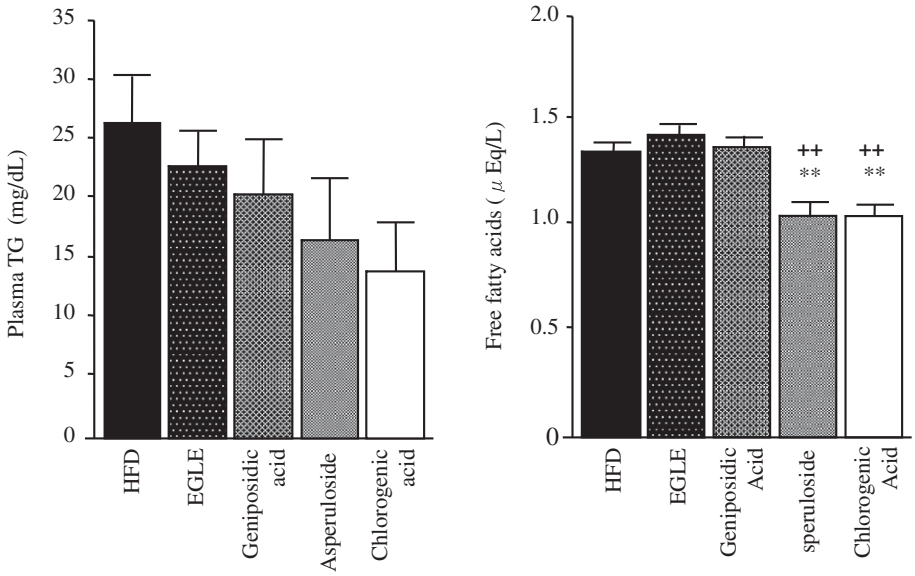

Figure 5. Effects of EGLE and three compounds on plasma TG and Free fatty acids in HFD-fed mice. Each value represent the mean ± S.E. Significantly different from HFD, $^{**}p$ <0.01 (Tukey *t*-test). Significantly different from EGLE, ^{++}p <0.01 (Tukey *t*-test).

of asperuloside on adipocytes and adipocytokines. Further research in this area may enable use of asperuloside in the treatment of obesity.

In conclusion, these findings suggest that administration of Eucommia leaf and the 30% MeOH fraction which contains more asperuloside, give anti-obesity effects, such as decrease in the body weight, WAT weight and plasma levels of triglyceride and total cholesterol. These effects might depend on asperuloside content in Eucommia leaf, since chronic administration of asperuloside decreased the body weight, WAT weight and the plasma lipid parameters in mice fed a HFD. This suggests that asperuloside in Eucommia leaves has anti-obesity effect.

Acknowledgments

The authors are grateful to Dr. S. Nishibe (Health Sciences University of Hokkaido, Japan) for helpful suggestions.

Supplementary data

Supplementary data (male SD rats (4 weeks old; 75–80 g) purchased from SLC Corporation (Shizuoka, Japan) were maintained at a temperature of 23–26 °C and relative humidity of 50–65% for 2 weeks after arrival. Rats were divided into groups ($n = 4$) based on body weight. The experimental diet consisted of 30% lard on MF diet (HFD: Oriental Yeast Co., Ltd, Tokyo, Japan). Test materials were prepared by adding 5% ELE, 0.1% geniposidic acid, 0.1% asperuloside, and 0.1% chlorogenic acid. After 30 days of administration, respiratory metabolism was measured using the MK-5000RQ CO_2/O_2 metabolism measuring system for small animals (Muromachi Kikai Co., Ltd, Tokyo, Japan) over 20 h. The respiratory quotient (RQ = CO_2 extrusion/O_2 consumption) and basal metabolic rate (BMR = (VO_2 mL/min)/body weight (kg)$^{0.75}$) were calculated for each group) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.060.

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12. The fresh leaves of *Eucommia ulmoides* Oliv. collected in August 2005 at Chengdu, Sichuan Province, People's Republic of China, were momentarily treated with steam at 100–110 °C, and then, dried. A voucher specimen (No. 200) was identified by T.N. and deposited in the Herbarium of the Department of Natural Medicines, Kumamoto University, Kumamoto, Japan. Dried leaves (20 kg) of *Eucommia ulmoides* were extracted with hot water for 10 h at 60 °C, vacuum-dried, and powdered to obtain EGLP (3.6 kg). Five fractions were prepared as follows. The extract was subjected to Diaion HP-20P column chromatography with a gradient of H_2O–MeOH (1:0–0:1) to yield H_2O fractions (Fr. 1; 4.52 kg), 30% MeOH fractions (Fr. 2; 432 g), 50% MeOH fractions (Fr. 3; 215 g), 80% MeOH fractions (Fr. 4; 76.7 g) and 100% MeOH fractions (Fr. 5; 2.4 g), in the order of elution.
13. Female ICR mice (4 weeks old; 23–28 g) purchased from SLC, Inc. (Shizuoka, Japan) were maintained at a temperature of 23–26 °C, relative humidity of 40–60%, and a 12-h light-dark cycle (lights on at 8 AM and off at 8 PM) for 1 week after arrival. Mice were divided into groups ($n = 8$) based on body weight. The experimental diet consisted of 40% beef tallow, 36% casein, 9% granulated sugar, 4% mineral mixture, 1% vitamin mixture, 10% cornstarch, and test materials (beef tallow, casein, granulated sugar, mineral mixture, vitamin mixture, cornstarch; Oriental Yeast Co., Ltd, Tokyo, Japan). The high-fat diet (HFD) group received feed in which one of the test materials was replaced by 10% casein.
14. For determination of the major components (geniposidic acid, asperuloside and chlorogenic acid) in the samples, an HPLC system comprised of a YMC-Pack ODS column (YMC Co., Ltd, Kyoto, Japan; 6.0 × 150 mm, 5 μm), LC-10AT pumps, and a SPD-10A UV detector (Shimazu Co., Ltd, Kyoto, Japan) was assembled. The mobile phase was methanol/water/phosphoric acid (870:130:1, v/v) at a flow rate of 1.0 ml/min. The detection wavelength was 215 nm.

15. Test materials were prepared by adding 10% EGLP, 7.97% Fr. 1, 0.76% Fr. 2, 0.38% Fr. 3, 0.14% Fr. 4 and 0.004% Fr. 5.
16. After 4 weeks of administration, body weight was measured and mice were sacrificed by decapitation without loading stress. Blood was collected immediately after the animals had been sacrificed by decapitation. The blood was centrifuged (3000 rpm, 30 min), and the separated plasma was stored at −80 °C until measurement. The levels of plasma TG and plasma total cholesterol were measured using commercially available enzyme kits (triglyceride: E-Test Wako, cholesterol: E-Test Wako; Wako Pure Chemicals Industries, Ltd, Osaka, Japan). Levels of FFA were measured using commercially available enzyme kits (FFA kit: Wako Pure Chemicals Industries, Ltd, Osaka, Japan). White adipose tissues (WAT) (abdominal visceral fat) were immediately removed. All procedures were carried out in accordance with the Guidelines for Animal Experiments at Kobayashi Pharmaceutical Co., Ltd, R & D Center, the Japanese Government Animal Protection and Management Law (No. 105), and the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).
17. Differences between groups were assessed with the unpaired Tukey *t*-test using a statistical package from the SPSS13.0 program. Differences were considered significant at $p < 0.05$.
18. (a) Yamauchi, T.; Kamon, J.; Waki, H.; Murakami, K.; Motojima, K.; Komeda, K.; Ide, T.; Kubota, N.; Terauchi, Y.; Tobe, K.; Miki, H.; Tsuchida, A.; Akanuma, Y.; Nagai, R.; Kimura, S.; Kadowaki, T. *J. Biol. Chem.* **2001**, *276*, 1796; (b) Yamauchi, T.; Kamon, J.; Waki, H.; Terauchi, Y.; Kubota, N.; Hara, K.; Mori, Y.; Ide, T.; Murakami, K.; Tsuboyama-Kasaoka, N.; Ezaki, O.; Akanuma, Y.; Gavrilova, O.; Vinson, C.; Reitman, M. L.; Kagechika, H.; Shudo, K.; Yoda, M.; Nakano, Y.; Tobe, K.; Nagai, R.; Kimura, S.; Tomita, M.; Froguel, P.; Kadowaki, T. *Nat. Med.* **2001**, *7*, 941; (c) Yamauchi, T.; Kamon, J.; Minokoshi, Y.; Ito, Y.; Waki, H.; Uchida, S.; Yamashita, S.; Noda, M.; Kita, S.; Ueki, K.; Eto, K.; Akanuma, Y.; Froguel, P.; Foufelle, F.; Ferre, P.; Carling, D.; Kimura, S.; Nagai, R.; Kahn, B. B.; Kadowaki, T. *Nat. Med.* **2002**, *8*, 1288.
19. A portion (1.0 g) of fraction 1 (135.5 g) was chromatographed over MCI gel CHP 20P with H_2O and MeOH, adjusted pH 3.5 with rare hydrochloric acid, and then purified by Daisogel SP-120-40/60 ODS-B (200 × 1000 mm I.D.) eluted with 50% MeOH to yield geniposidic acid (86.0 g, purity 98.9%). Asperuloside was prepared as follows. Fr. 2 (270 g) was chromatographed over YMC S-15/30 120A ODS with a gradient of H_2O–MeOH (1:1–6:1) and then purified by Daisogel SP-120-40/60-ODS-B (100 × 1000 mm) eluted with 80% MeOH, vacuum-dried and freeze-dried, and washed with acetone to furnish asperuloside (31.3 g, purity 99.5%). Geniposidic acid and asperuloside were extracted and isolated from EGLP.
20. Test materials were prepared by adding 0.63% geniposidic acid, 0.45% asperuloside, and 0.44% chlorogenic acid.
21. Cho, A. S.; Jeon, S. M.; Kim, M. J.; Yeo, J.; Seo, K. I.; Choi, M. S.; Lee, M. K. *Food Chem. Toxicol.* **2010**, *48*, 937.