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Anti-obesity effects of the methanolic extract and chakasaponins from the flower buds of *Camellia sinensis* in mice

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ABSTRACT

The methanolic extract from the flower buds of *Camellia sinensis* cultivated in Fujian Province showed inhibitory effects on body weight gain and the weight of visceral fats in high-fat diet-fed mice and/or Tsumura Suzuki Obese Diabetic (TSOD) mice. A suppressive effect of the extract on food intake was suggested to contribute to the anti-obesity effect. The *n*-butanol (BuOH)-soluble fraction also reduced food intake in normal diet-fed mice. A principal constituent, chakasaponin II, inhibited gastric emptying (GE) as well as food intake. These inhibitory effects were partly reduced by pretreatment with a high dose of capsaicin. The *n*-BuOH-soluble fraction and chakasaponin II suppressed mRNA levels of neuropeptide Y (NPY), an important regulator of body weight through its effects on food intake and energy expenditure, in the hypothalamus. Furthermore, chakasaponin II enhanced the release of serotonin (5-HT) from the isolated ilea of mice in vitro. These findings suggested that the active saponins suppressed the appetite signals in the hypothalamus through stimulation of the capsaicin-sensitive sensory nerves, probably vagal afferent nerves, or enhancement of 5-HT release from the ilea, leading to reduced food intake and body weight gain.

1. Introduction

The flower buds of *Camellia sinensis* (Tea Flower, 'Chaka' in Japanese) have been used as a food garnish in Japanese-style dishes: for example, 'Botebotecha' in Shimane prefecture. However, the chemical constituents and pharmacological properties of these flower buds have not yet been clarified. In our studies on the bioactive constituents of medicinal flowers, we have reported the isolation and structural elucidation of acylated polyhydroxyoleanane-type triterpene oligoglycosides, floratheasaponins A–L from the flower buds of *C. sinensis* cultivated in Japan and in Anhui Province, China,[1–5] and chakasaponins I–III (**1**–**3**), V, and VI from the flower buds of *C. sinensis* cultivated in Fujian Province, China.[6] These saponins showed anti-hyperlipidemic, anti-hyperglycemic, anti-allergic, and gastroprotective effects.[1–6]

In this paper, we describe the anti-obesity effects of the methanolic (MeOH) extract from the flower buds of *C. sinensis* cultivated in Fujian Province, China and its active principles and mode of action.

2. Results and discussion

2.1. Effects of the MeOH extract on body weight in high-fat diet-fed mice

We first examined the effects of the MeOH extract on body weight gain and the weight of visceral fat in a high-fat diet-fed mice. The MeOH extract (500 mg/kg, po/d) markedly inhibited body weight gain 9–14 d after the administration (Fig. 1A). The MeOH extract (250 and/or 500 mg/kg, po/d) significantly suppressed liver weight, liver triglyceride (TG) and the weight of visceral fat (Table 1). A positive control, bezafibrate (50 and 100 mg/kg, po/d), significantly reduced body weight gain [43.4 ± 0.9 g (p <0.05) at 50 mg/kg and 43.0 ± 1.1 g (p <0.05) at 100 mg/kg vs 46.2 ± 0.5 g (control group)] on day 14 after fasting. It also reduced weight of visceral fat and plasma TG at 100 mg/kg. Peroxisome proliferator-activated receptor (PPAR)α agonists including bezafibrate are reported to increase liver weight at higher doses in rats.[7] Consistent with this report, an increase in liver weight was observed in bezafibrate-treated mice. But, the MeOH extract did not show such an effect, and this result together with no effects on plasma TG levels suggested that the extract might not act as a PPARα agonist.

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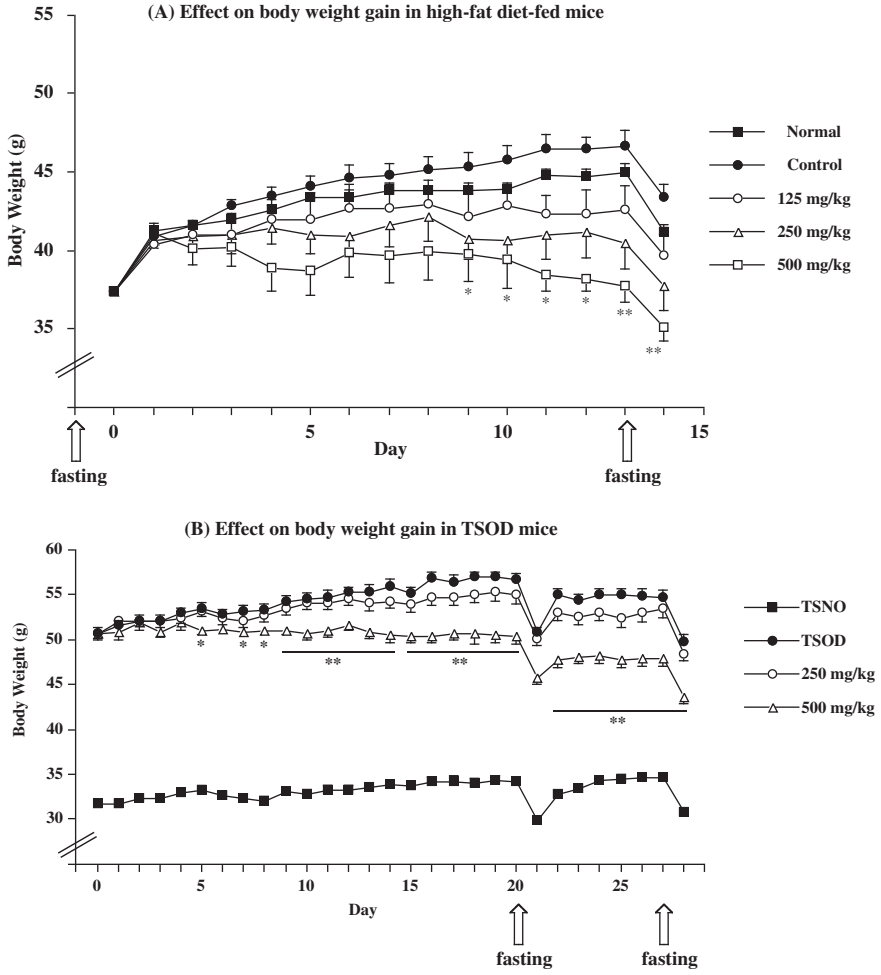


Figure 1. Effects of the MeOH extract on body weight gain in high-fat diet-fed mice and TSOD mice. (A) Male ddY mice were fed a high-fat diet (45 kcal% fat) or normal diet (10 kcal% fat) for 14 d. The test sample was given orally once a day. (B) TSOD and TSNO mice were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd) for 28 d. The test sample was given orally once a day. Each value represents the mean with SEM (n = 6–10). Significantly different from the control, $^{*}p$ <0.05; $^{**}p$ <0.01.

Table 1
Effects of the MeOH extract on liver weight, liver TG, plasma glucose and TG levels and weight of visceral fat in high-fat diet-fed mice

Treatment	Dose (mg/kg, po)	n	Liver (g)	Liver TG (mg/g wet tissue)	Plasma glucose (mg/dL)	Plasma TG (mg/dL)
Normal	—	7	1.31 ± 0.05	39.2 ± 4.4*	107.9 ± 6.1**	90.3 ± 14.4
Control	—	8	1.23 ± 0.03	62.1 ± 4.7	162.0 ± 15.2	128.3 ± 10.9
MeOH ext.	125	6	1.13 ± 0.06	49.0 ± 7.2	169.9 ± 9.6	106.8 ± 10.2
	250	7	1.09 ± 0.04	39.9 ± 5.9*	147.9 ± 13.6	110.4 ± 10.6
	500	7	1.06 ± 0.03*	30.2 ± 4.7**	147.7 ± 8.3	111.0 ± 10.8
Normal	—	7	1.46 ± 0.02	46.4 ± 5.3	116.3 ± 3.9*	99.9 ± 7.9
Control	—	8	1.42 ± 0.02	55.6 ± 8.5	142.3 ± 10.3	102.4 ± 14.2
Bezafibrate	50	5	2.45 ± 0.11**	44.0 ± 5.1	175.1 ± 5.9*	82.6 ± 3.9
	100	7	2.61 ± 0.09**	43.3 ± 6.6	155.6 ± 4.5	60.1 ± 4.3*
	Dose (mg/kg, po)	n	Mensenteric fat (g)	Epididymal fat (g)	Nephritic fat (g)	Visceral fat[a] (g)
Normal	—	7	0.55 ± 0.08	1.17 ± 0.16	0.49 ± 0.06	2.22 ± 0.29
Control	—	8	0.65 ± 0.06	1.44 ± 0.18	0.64 ± 0.06	2.73 ± 0.27
MeOH ext.	125	6	0.60 ± 0.07	1.28 ± 0.16	0.60 ± 0.07	2.47 ± 0.28
	250	7	0.56 ± 0.10	1.25 ± 0.20	0.58 ± 0.09	2.39 ± 0.37
	500	7	0.41 ± 0.05	0.86 ± 0.10	0.43 ± 0.07	1.70 ± 0.21*
Normal	—	7	0.50 ± 0.07	1.10 ± 0.14	0.45 ± 0.08	2.05 ± 0.28
Control	—	8	0.70 ± 0.05	1.45 ± 0.11	0.66 ± 0.07	2.81 ± 0.20
Bezafibrate	50	5	0.54 ± 0.03	1.20 ± 0.06	0.51 ± 0.04	2.25 ± 0.08
	100	7	0.50 ± 0.08	0.96 ± 0.16	0.45 ± 0.06	1.92 ± 0.29*

Each value represents the mean ± SEM.
[a] The weight of visceral fat was estimated as the total weight of mesenteric, epididymal, and nephritic fats.
* p <0.05, significantly different from the control group.
** p <0.01, significantly different from the control group.

2.2. Effects of the MeOH extract on body weight gain, weights of liver and visceral fat, and a glucose tolerance test in TSOD mice

Next, the effect of the MeOH extract in an experimental animal of metabolic syndrome, Tsumura Suzuki Obese Diabetic (TSOD) mice,[8] was examined. As shown in Figure 1B, the extract (500 mg/kg, po/d) also significantly suppressed body weight gain after a week. Three weeks later, a glucose tolerance test (GTT) by intraperitoneal injection of glucose was examined. The MeOH extract (250 and 500 mg/kg, po/d) significantly suppressed the increase in plasma glucose levels 2 h after glucose loading (Table 2). After 4 weeks, liver weight, weight of visceral fat, and plasma total cholesterol levels were significantly suppressed by the extract (500 mg/kg, po/d) (Table 3).

2.3. Effects of the MeOH extract on food intake in high-fat diet-fed mice, TSOD mice, and normal mice

Next, the effect of the extract on food intake was examined in high-fat diet-fed mice and TSOD mice. As shown in Figure 2A and B, the extract inhibited food intake in a dose-dependent manner. Furthermore, this effect was also observed in normal diet-fed mice; the total intake for 5 d in the MeOH-treated group (500 mg/kg, po/d) was 19.3 g (p <0.01) versus 21.0 g in the control group (n = 6), but an obvious toxic effect was not observed (data not shown) except for body weight gain.

2.4. Effects of the *n*-butanol (*n*-BuOH)-soluble fraction on food intake and neuropeptide Y (NPY) and Agouti-related protein (AgRP) mRNA levels in hypothalamus

As shown in Figure 3A, the n-BuOH-soluble fraction inhibited food intake at a dose of 250 mg/kg, po/d, but the ethyl acetate (EtOAc)- and H_2O-soluble fractions had no such effect; the total intake for 4 d in the EtOAc (50 mg/kg, po/d)- and H_2O (200 mg/kg, po/d)-soluble fractions-treated groups was 14.9 and 15.9 g/mouse versus 15.5 g/mouse in the control group, when the fraction was given orally according to its yield.

With regard to the effect of the n-BuOH-soluble fraction on appetite signals, effects on NPY and AgRP mRNA levels in the hypothalamus were examined. NPY is an important regulator of body weight through its effects on food intake and energy expenditure. The majority of neurons expressing NPY in the hypothalamus are found within the arcuate nucleus (ARC) and most co-express AgRP. The ablation of NPY/AgRP neurons in young mice reduces food intake and body weight, and the icv injection of NPY potently stimulates food intake in adult rats.[9] In the present study, the n-BuOH-soluble fraction administrated at 250 mg/kg for 4 d significantly suppressed the expression of NPY mRNA, although AgRP mRNA levels were not significantly reduced (Table 4). These findings suggested that the n-BuOH-soluble fraction inhibited food intake by suppressing appetite signals.

2.5. Effect of principal constituents, chakasaponin II (2) and a flavonol glycoside 5 on food intake in mice

From the active fraction (n-BuOH-soluble fraction), we previously isolated new acylated polyhydroxy-oleanane-type triterpene oligoglycosides, chakasaponins I–III (**1**–**3**), V, and VI and known flavanoids, and elucidated their chemical structures based on chemical and physicochemical evidence.[6] In the present study, effects of a principal saponin, chakasaponin II (**2**), and a principal flavonol glycoside, kaempferol 3-*O*-β-D-glucopyranosyl(1-3)-α-L-rhamnopyranosyl(1-6)-β-D-glucopyranoside (**5**) (Fig. 4), on food intake were examined.

Chakasaponin II (**2**) caused a similar suppression of food intake at 50 mg/kg, po/d (Fig. 3B).[10] But **5** did not show such an effect; the total food intake for 7 d in the **5** (50 mg/kg, po/d)-treated group was 29.2 g/mouse versus 30.1 g/mouse in the control group. Furthermore, **2** at 50 mg/kg, po/d significantly inhibited NPY mRNA levels in hypothalamus similar to the n-BuOH-soluble fraction (Table 4). These results suggest that the saponins are active constituents of the extract. Furthermore, the desacyl derivative of **2**, desacyl-floratheasaponin B (**4**), lacked the effect (Fig. 3C), suggesting that the 21,22-acyl groups are important for the activity.

Furthermore, the effects of the n-BuOH-soluble fraction and chakasaponin II (**2**) on food intake were obviously reduced in the capsaicin-pretreated mice (Fig. 3A and B). These findings suggested that their inhibitory effects on food intake were mediated through the capsaicin-sensitive sensory nerves, probably, vagal afferent nerves.

Recently, an anti-cancer drug, cisplatin, and selective serotonin reuptake inhibitors (SSRIs) were reported to inhibit food intake, and the involvement of 5-HT_2 receptors in appetite control has been shown. Appetite is suppressed when the 5-HT_{2B} receptor in gastric smooth muscle and the 5-HT_{2C} receptor in the hypothalamus are activated. 5-HT produced during treatment with cisplatin or SSRIs binds to various receptor subtypes and is likely to stimulate the 5-HT_{2B} and 5-HT_{2C} receptors. The stimulation of 5-HT_{2B} receptor decreases plasma ghrelin levels, and suppresses the appetite signals via afferent vagal nerves.[11–13] Consistent with previous reports, 5-HT (serotonin creatinine sulfate monohydrate, 1 mg/kg, ip) inhibited the food intake in mice (Fig. 3D).

2.6. Effects of the MeOH extract, the *n*-BuOH-soluble fraction, and its principal constituents, chakasaponins I (1) and II (2), on gastric emptying

We previously reported that several saponins with similar structures, theasaponins from the seeds of *C. sinensis* and escins

Table 2
Effects of the MeOH extract on glucose tolerance test in TSOD mice

Treatment	Dose (mg/kg, po)	n	Increase in plasma glucose levels (Δmg/dL)		
			0.5 h	1.0 h	2.0 h
Normal (TSNO)	—	10	251.5 ± 8.4*	216.3 ± 14.4**	93.9 ± 8.3**
Control (TSOD)	—	7	303.4 ± 8.5	330.5 ± 12.4	305.7 ± 13.5
MeOH ext.	250	7	289.7 ± 16.0	286.8 ± 17.8	243.0 ± 16.2*
	500	6	277.8 ± 22.4	280.5 ± 19.3	203.3 ± 24.3**

On day 21, the test sample was given orally to the 20 h-fasted mice. One hour later, glucose was given (2.0 g/kg, ip). Blood samples were collected at 0.0, 0.5, 1.0, and 2.0 h after loading of glucose. The plasma glucose levels of normal group, control group, and the MeOH extract-treated groups (250 and 500 mg/kg) at 0 h were 124.4 ± 3.2, 145.4 ± 3.4, 198.2 ± 5.0, and 191.2 ± 15.7, respectively.
Each value represents the mean ± SEM of increase in plasma glucose levels compared to the levels just before the glucose loading (0 h).
* p <0.05, significantly different from the control group.
** p <0.01, significantly different from the control group.

Table 3
Effects of the MeOH extract on liver weight, liver TG, plasma glucose, TG, and total cholesterol levels and weight of visceral fat in TSOD mice

Treatment	Dose (mg/kg, po)	*n*	Liver (g)	Liver TG (mg/g wet tissue)	Plasma glucose (mg/dL)	Plasma TG (mg/dL)	Plasma total cholesterol (mg/dL)
Normal (TSNO)	—	10	1.17 ± 0.03**	50.9 ± 4.6**	127.0 ± 4.2**	40.2 ± 5.0**	161.9 ± 4.8
Control (TSOD)	—	7	1.48 ± 0.04	23.8 ± 2.8	155.8 ± 6.1	142.3 ± 7.6	254.3 ± 13.1
MeOH ext.	250	7	1.39 ± 0.05	22.6 ± 1.6	159.1 ± 9.5	159.8 ± 10.2	223.6 ± 9.0
	500	6	1.27 ± 0.03**	19.5 ± 2.2	142.9 ± 10.2	123.6 ± 19.3	210.4 ± 15.0*

	Dose (mg/kg, po)	*n*	Mensenteric fat (g)	Epididymal fat (g)	Nephritic fat (g)	Visceral fata (g)
Normal	—	10	0.34 ± 0.02**	0.49 ± 0.02**	0.21 ± 0.01**	1.04 ± 0.05**
Control	—	7	1.72 ± 0.08	2.15 ± 0.03	1.36 ± 0.07	5.23 ± 0.14
MeOH ext.	250	7	1.72 ± 0.08	2.10 ± 0.08	1.37 ± 0.11	5.18 ± 0.25
	500	6	1.04 ± 0.08**	1.79 ± 0.07**	0.85 ± 0.07**	3.67 ± 0.18**

Each value represents the mean ± SEM.
[a] The weight of visceral fat was estimated as the total weight of mesenteric, epididymal, and nephritic fats.
* p <0.05, significantly different from the control group.
** p <0.01, significantly different from the control group.

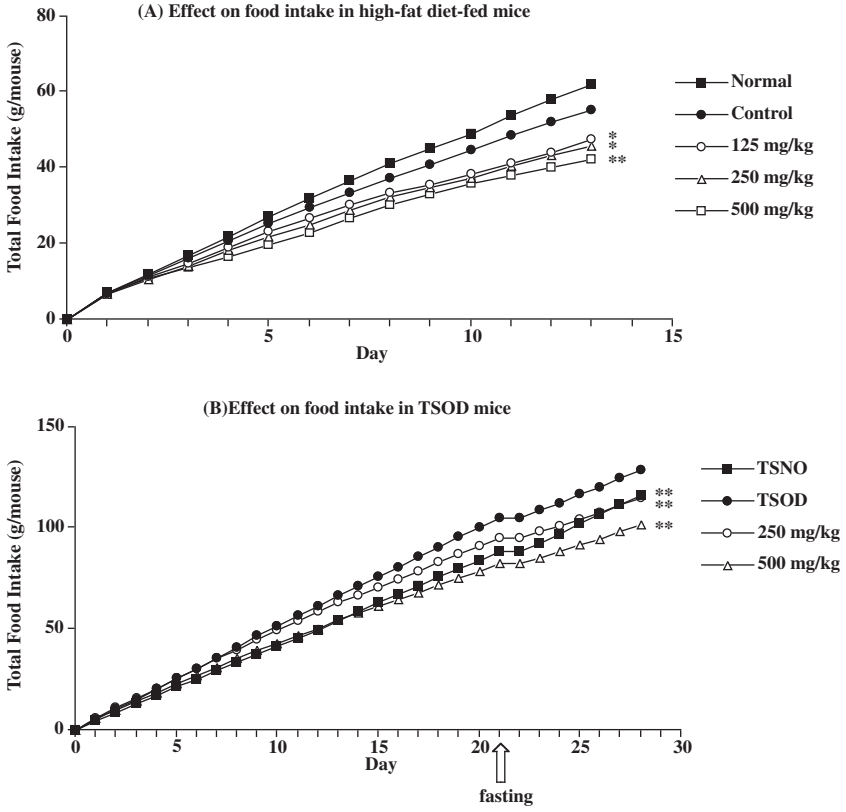


Figure 2. Effects of the MeOH extract on food intake in high-fat diet-fed mice and TSOD mice. (A) Male ddY mice were fed a high-fat diet (45 kcal% fat) or normal diet (10 kcal% fat) for 14 days. The test sample was given orally once a day. (B) TSOD and TSNO mice were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd) for 28 d. The test sample was given orally once a day. Each value represents the mean for 6–10 mice. Significantly different from the control, *p <0.05; **p <0.01.

from the seeds of *Aesculus turbinata*, showed anti-hyperlipidemic effects in olive oil- or a lipid emulsion-loaded mice,[1,14] and inhibition of pancreatic lipase activity was suggested to be partly involved in their effect.[6,14] In addition, their anti-hyperlipidemic effects are considered to mainly depend on the inhibition of gastric emptying.[15,16] We, therefore, examined the effects of the MeOH extract and its *n*-BuOH-soluble fraction on gastric empting in mice. Thirty minutes after the oral administration of CMC-Na containing phenol red, the amount of phenol red in the stomach was determined.

As shown in Table 5, the MeOH extract and *n*-BuOH-soluble fraction significantly inhibited the gastric emptying in a dose-dependent manner. Furthermore, we examined effects of principal saponins, chakasaponins I (**1**) and II (**2**), on the gastric emptying. Both compounds **1** and **2** (25 and 50 mg/kg) also inhibited the gastric emptying in mice.

Previously, we reported that the inhibitory effects of escins on gastric emptying involved the release of dopamine and dopamine$_2$ receptors and capsaicin-sensitive sensory nerve-, probably vagal afferent nerve-, mediated mechanisms.[17] Tominaga et al. recently reported that 5-HT inhibited gastric emptying in rats.[18] Consistent with the previous report, 5-HT (serotonin creatinine sulfate monohydrate, 10 mg/kg, ip) significantly inhibited gastric emptying under our experimental conditions, but effective dose of 5-HT was higher than that of effects on food intake.

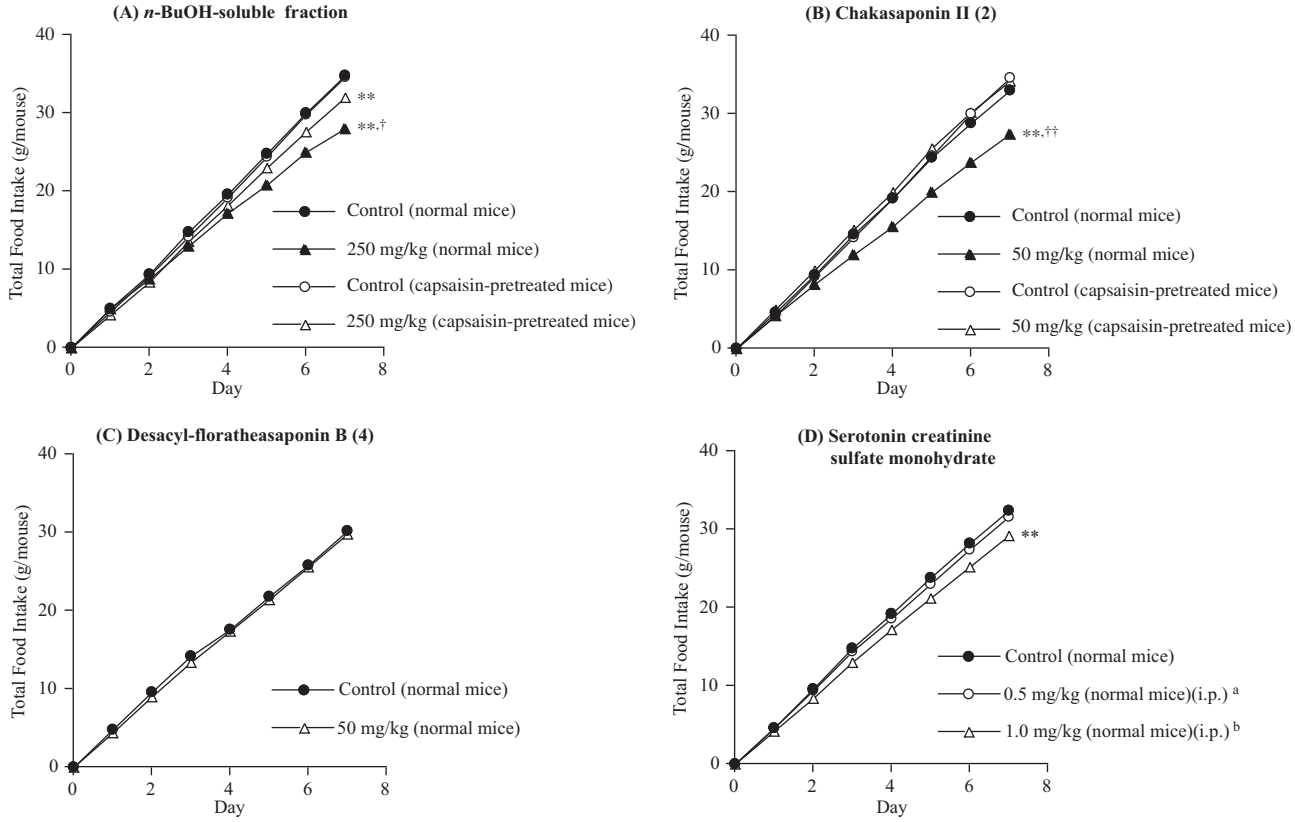


Figure 3. Effects of the BuOH-soluble fraction, chakasaponin II (**2**), desacyl-floratheasaponin (**4**), and 5-HT on food intake in normal mice and/or capsaicin-pretreated mice Male ddY mice were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd) for 8 d. The test sample was given orally once a day. [a,b]These doses are equivalent to ca. 0.2 and 0.4 mg/kg serotonin. Each value represents the mean for 5 or 6 mice. Significantly different from the control, $^{**}p$ <0.01, and from the corresponding capsaicin-treated group, $^{\dagger}p$ <0.05, $^{\dagger\dagger}p$ <0.01.

Table 4
Effect of the *n*-BuOH-soluble fraction and chakasaponin II (**2**) on levels of NPY and AgRP mRNA in hypothalamus of mice

Treatment	Dose (mg/kg, po)	*n*	Target/β-actin ratio	
			NPY	AgRP
Control	—	6	1.00 ± 0.13	1.00 ± 0.10
n-BuOH-soluble fraction	125	6	0.98 ± 0.08	0.98 ± 0.08
	250	6	0.61 ± 0.07*	0.92 ± 0.04
Control	—	6	1.00 ± 0.13	1.00 ± 0.24
Chakasaponin II (**2**)	25	6	1.09 ± 0.04	1.08 ± 0.13
	50	6	0.63 ± 0.06**	1.03 ± 0.20

The test sample was given orally to male ddY mice once a day. Four days later, the hypothalamus was dissected out, and the NPY and AgRP mRNA levels were determined.
Each value represents the mean ± SEM.
* p <0.05, significantly different from the control group.
** p <0.01, significantly different from the control group.

2.7. Effects of the *n*-BuOH-soluble fraction and/or chakasaponins II (2) on release of 5-HT in isolated mouse ilea

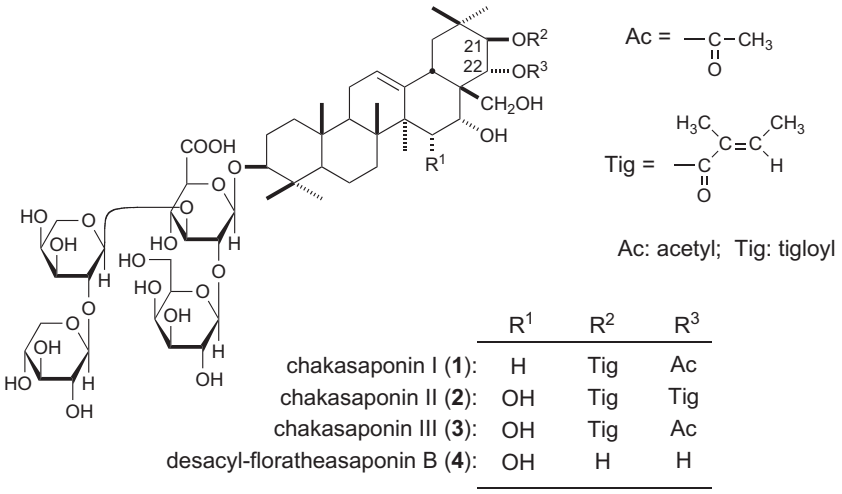
Next, we examined the release of 5-HT from isolated ilea and its retention in the tissues. Chakasaponin II (**2**) at 1000 μM significantly enhanced the release of 5-HT into the medium and reduced its retention in the tissues (Table 6).[19] This concentration is relatively high, but the concentrations of the saponin in the intestinal tract are thought to be relatively high, since this type of compound is reported to be difficult to absorb.[20]

2.8. Effects of the *n*-BuOH soluble-fraction and chakasaponins I (1) and/or II (2) on food intake and gastric emptying in capsaicin-pretreated mice

As described in Section 2.6, the inhibitory effects of escins on gastric emptying involved the capsaicin-sensitive sensory nerves, probably, vagal afferent nerves.[15] To confirm the involvement of the capsaicin-sensitive sensory nerves, we examined effects of the *n*-BuOH-soluble fraction and chakasaponins I (**1**) and/or II (**2**) on food intake and gastric emptying in mice in which the capsaicin-sensitive sensory nerves were denerved by pretreatment with a high dose of capsaisin. As shown in Figure 3A and B, pretreatment with capsaicin reduced the inhibitory effects of the *n*-BuOH-soluble fraction and chakasaponin II (**2**) on food intake. Furthermore, pretreatment with capsaicin partly reduced the inhibitory effects of the *n*-BuOH-soluble fraction and chakasaponins I (**1**) and II (**2**) on gastric emptying (Table 5). These findings suggest that the capsaicin-sensitive sensory nerves, probably vagal afferent nerves, are involved in the inhibition of food intake similar to that of gastric emptying, at least in part. Bugajski et al. reported that long-term vagal electrical stimulation reduced food intake and body weight in rats.[21] Therefore, in addition to 5-HT release, other mechanisms of action including the direct stimulation of vagal nerves by the saponins should be studied further.

3. Conclusion

In conclusion, the MeOH extract from the flower buds of *C. sinensis* cultivated in Fujian Province showed inhibitory effects



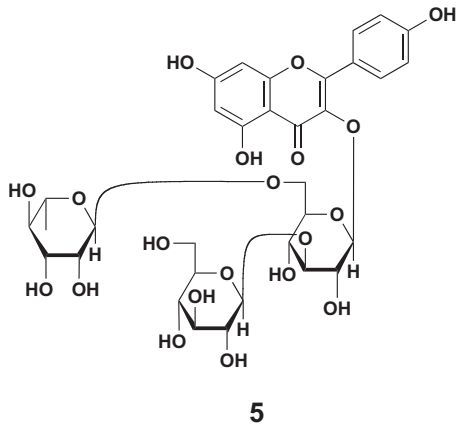


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Figure 4. Chemical structures of **1**–**5**.

Table 5
Inhibitory effects of the MeOH extract, the *n*-BuOH-soluble fraction, and chakasaponins I (**1**) and II (**2**) on gastric emptying in normal mice or in capsaicin-pretreated mice

Treatment	Dose (mg/kg, po)	Gastric emptying (%)			
		n	Normal mice	*n*	Capsaicin-pretreated mice
Control	—	9	82.2 ± 1.6		
MeOH ext.	125	8	70.8 ± 4.9**		
	250	8	59.1 ± 2.6**		
	500	8	41.4 ± 3.1**		
Control	—	6	85.8 ± 2.0	6	88.6 ± 1.8
n-BuOH-soluble fraction	125	6	50.8 ± 2.5**	6	72.9 ± 4.0**,††
	250	6	40.0 ± 2.3**	6	43.9 ± 3.0**
Control	—	6	86.6 ± 1.8	6	85.8 ± 2.0
Chakasaponin I (**1**)	25	6	76.4 ± 5.4	6	79.2 ± 3.0
	50	6	55.7 ± 1.3**	6	68.0 ± 4.4**,†
Control	—	6	90.4 ± 1.2	6	87.1 ± 2.3
Chakasaponin II (**2**)	25	6	77.9 ± 2.7	6	81.4 ± 2.2
	50	6	57.6 ± 5.9**	6	67.1 ± 2.3**
Control	—	8	87.9 ± 1.6		
Serotonin creatinine	5 (ip)[a]	8	76.9 ± 4.8		
Sulfate monohydrate	10 (ip)[a]	8	53.7 ± 2.5**		

Each value represent the mean ± S.E.M.
Significantly different from the control group, $**p<0.01$, and from corresponding capsaicin-untreated group (normal mice), $†p<0.05$, $††p<0.01$.
[a] These doses are equivalent to *ca.* 2 and 4 mg/kg serotonin.

on body weight gain and the weight of visceral fat in high-fat diet-fed mice and TSOD mice. From the active fraction (*n*-BuOH-soluble fraction), an acylated polyhydroxyoleanane-type triterpene oligoglycoside, chakasaponin II (**2**), was isolated as an active constituent. With regard to anti-obesity effects, the BuOH-soluble fraction and **2** reduced food intake in high-fat diet- and normal

Table 6
Effects of chakasaponin II (**2**) on 5-HT release from isolated ileum in mice

Treatment	Dose (μM)	*n*	5-HT release into the medium (ng/g tissue)	5-HT remained in the tissue	
				(ng/mg protein)	(ng/g tissue)
Control	—	8	11.2 ± 1.3	0.861 ± 0.164	68.8 ± 12.4
Chakasaponin II (**2**)	100	8	15.6 ± 1.9	0.659 ± 0.115	50.8 ± 7.6
	1000	8	28.3 ± 2.8**	0.396 ± 0.084*	31.6 ± 6.9*

Ileum segments (0.03–0.05 g) of mice were incubated in 1 mL of modified Krebs's solution with or without test sample. After incubation for 20 min, 5-HT levels in the medium and tissues were determined using HPLC with an ECD.
Each value represents the mean ± SEM.
* p <0.05, significantly different from the control group.
** p <0.01, significantly different from the control group.

diet-fed mice and on gastric emptying (GE) in mice. The *n*-BuOH-soluble fraction and **2** suppressed NPY mRNA levels in the hypothalamus, suggesting the suppression of an appetite signal. The inhibitory effects of the *n*-BuOH fraction and **1** and/or **2** on food intake and GE were partly reduced by pretreatment with a high-dose of capsaicin. Furthermore, **2** enhanced the release of 5-HT from the isolated ilea of mice in vitro. These findings suggested that the active saponins suppressed the appetite signals in the hypothalamus through stimulation of the capsaicin-sensitive sensory nerves, probably vagal afferent nerves, or enhancement of 5-HT release from the ilea, leading to reduced food intake and body weight gain. To the best of our knowledge, this is the first report regarding to the appetite reducing effects of saponins.

4. Materials and methods

4.1. Materials

The methanolic extract and isolation of chakasaponins I–III (**1**–**3**) and kaempferol 3-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**5**) were described in our previous paper.[6] Briefly, flower buds of *C. sinensis* (1.5 kg) cultivated in Fujian Province were treated with methanol to obtain the MeOH extract (31.1% from the material). The MeOH extract was partitioned into an EtOAc/H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (3.2%) and aqueous layer. The aqueous layer was further extracted with *n*-BuOH to give *n*-BuOH- (16.4%) and H_2O- (11.5%) soluble fractions. The *n*-BuOH-soluble fraction was subjected to normal-phase and reversed-phase silica gel column chromatographies and repeated HPLC to give chakasaponins I (**1**, 0.47%), II (**2**, 0.66%), and III (**3**, 0.13%) together with kaempferol 3-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (**5**, 0.36%), (−)-epigallocatechin 3-*O*-gallate (0.21%), (−)-epicatechin 3-*O*-gallate (0.067%), (−)-epicatechin (0.000077%), benzyl β-D-glucopyranoside (0.00021%), (*S*)-1-phenylethyl-β-D-glucopyranoside (0.050%), and caffeine (0.067%). Desacyl-floratheasaponin B (**4**) was obtained by alkaline hydrolysis of chakasaponin II (**2**) as described in our previous report.[6]

4.2. Reagents

Serotonin creatinine sulfate monohydrate, capsaicin, Glucose C-II test Wako, Triglyceride E-test Wako, and Cholesterol E-test Wako were purchased from Wako Pure Chemical Industries. Co., Ltd (Osaka, Japan). The RNeasy™ mini Kit was from Qiagen. The ReverTra Ace® qPCR RT Kit and THUNDERBIRD™ SYBR® qPCR Mix were from Toyobo Co., Ltd.

4.3. Animals

Male ddY mice were purchased from Kiwa Laboratory Animal Co., Ltd (Wakayama, Japan). Male TSOD (Tsumura Suzuki Obese Diabetes) mice and male TSNO (Tsumura Suzuki Non-Obesity) mice were from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed at a constant temperature of 23 ± 2 °C and fed standard laboratory chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) unless otherwise noted. The experimental protocol was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

4.4. Effect on high fat diet-fed mice

Male ddY mice (10 w) were fed a normal diet (10 kcal% fat, D12450B; Research Diets, Inc.) or high-fat diet (45 kcal% fat, D12451; Research Diets, Inc) for 14 d. The test sample suspended in a 5% acacia solution was given orally by means of a metal orogastric tube once a day at 15:00–17:00. Body weight was measured daily. The mice were fasted for 20 h before the 1st and 14th days, and blood (ca. 0.2 mL) was collected from the infraorbital venous plexus under ether anesthesia. The blood was immediately mixed with heparin sodium (10 units/tube). After centrifugation of the blood sample, plasma glucose, and triglyceride (TG) levels were determined using commercial kits (Glucose CII-test Wako and Triglyceride E-test Wako). After 14 d, the mice were sacrificed by cervical dislocation under ether anesthesia, and visceral fat was weighed. The weight of visceral fat was estimated as the total weight of mesenteric, epididymal, and nephritic fats. Liver was also weighed and liver tissue (ca. 100 mg) was homogenized (TissueLyser, Qiagen) in 1 mL of water and TG levels were determined using a commercial kit (Triglyceride E-test Wako).

4.5. Effect on TSOD mice

Male TSOD mice and male TSNO mice (8 w old) were used. After the 2-week acclimation period, mice were housed and fed a chow diet (MF, Oriental Yeast Co., Ltd, Tokyo, Japan) for 28 d. The test sample suspended in a 5% acacia solution was given orally once a day at 15:00–17:00. Body weight was measured daily. The mice were fasted for 20 h before the 21st and 28th days, and a GTT was performed on day 21. On day 28, visceral fat (mesenteric, epididymal, and nephritic fats) and liver were weighed and liver TG levels were determined as described previously. Plasma glucose, TG, and total cholesterol concentrations were determined using commercial kits (Glucose CII-test Wako, Triglyceride E-test Wako, and Cholesterol E-test Wako).

4.6. Glucose tolerance test (GTT) in TSOD mice

The test sample was administered orally to 20–24 h-fasted mice on day 21. One hour thereafter, a 20 (w/v) % D-glucose solution (10 mL/kg) was administered intraperitoneally. Blood was collected from the infraorbital venous plexus under ether anesthesia at 0, 0.5, 1, and 2 h after the administration of D-glucose. Blood glucose levels were determined using a commercial kit (Glucose CII-test Wako).

4.7. Food intake

Male ddY mice (5 or 6 mice/cage) were housed and fed a standard laboratory chow. The test sample suspended in a 5% acacia solution was given orally once a day at 15:00–17:00. Food intake was determined every 24 h. Overall food intake (g/mouse) was then calculated. Food intake in the test sample-treated group for more than 4 d was compared to that of the control group, and statistical significance was calculated.

4.8. Gastric emptying in mice

The rate of gastric emptying was determined using a phenol red methods.[15] Briefly, a solution of 1.5% carboxymethyl cellulose sodium salt (CMC-Na) containing 0.05% phenol red as a marker was given intragastrically (0.3 mL/mouse) to 20–24 h-fated mice. Thirty minutes later, mice were sacrificed by cervical dislocation under ether anesthesia. The abdominal cavity was opened, the gastroesophageal junction and pylorus were clamped, and the stomach was removed, weighed, placed in 10 mL of 0.1 M NaOH, and homogenized. The suspension was allowed to settle for 1 h at room temperature, and 5 mL of the supernatant was added to 0.5 mL of 20% trichloroacetic acid (w/v) and centrifuged at 3000 rpm for 20 min. Next, 4 mL of supernatant was mixed with 4 mL of 0.5 M NaOH, and the amount of phenol red was determined from the absorbance at 560 nm. The test sample was given orally 30 min prior to the administration of the CMC-Na solution. Gastric emptying (%) in the 30-min period was calculated according to the following equation:

$$\text{gastric emptying } (\%) = (1 - \text{amount of phenol red in test sample-treated group/amount of administered phenol red}) \times 100$$

4.9. Capsaicin-pretreated mice

The capsaicin solution was prepared in a solution containing 99.5% ethanol, Tween 80, and saline (2:1:7, v/v/v). Mice were anesthetized with diethyl ether, and treated with capsaicin for 3 consecutive days (25 mg/kg, sc) to deplete neuropeptides in primary afferent neurons, as described previously.[15] To counteract any respiratory impairment associated with the administration of capsaicin, the mice were pretreated with aminophylline (10 mg/kg, dissolved in 5 mL saline, im) 30 min before receiving the capsaicin injection. After 14 d, the efficiency of capsaicin pretreatment was verified by a corneal chemosensory test, which consists of monitoring the wiping reflex to the ocular instillation of a drop of a 0.5% NH_4OH solution. None of the capsaicin-pretreated mice showed a wiping response, indicating the effective ablation of primary sensory afferents, whereas the wiping reflex was present in vehicle-pretreated mice.

4.10. Expression of neuropeptide Y (NPY) and Agouti-related protein (AgRP) mRNA in hypothalamus

The test sample was given orally to male ddY mice once a day at 15:00–17:00. Four days later, the hypothalamus was dissected 1 h after the final administration of the test sample. These tissues were stored in RNA later (Qiagen) for one night and kept at −80 °C until they were processed for RNA extraction. Isolated tissue was homogenized with TissueLyser (Qiagen). Total RNA was extracted from the cells using an RNeasy™ mini Kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and determining the ratio of the readings at 260 nm and 280 nm. cDNA was synthesized from 0.5 μg of total RNA using a ReverTra Ace® qPCR RT Kit (Toyobo Co., Ltd) according to the manufacturer's instructions. The template cDNA thus obtained was incubated with gene-specific primers and with THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Co., Ltd) in a MiniOpticon (Bio-Rad Laboratories). The abundance of each gene product was calculated by relative quantification, with values for the target genes normalized to β-actin mRNA. The thermal cycling program had an initial denaturation (95 °C for 2 min) and then 40 cycles of denaturation (95 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 30 s). The primer pairs were: NPY primers, 5'-CTGACCCTCGCTCTATCTCTG-3' and 5'-AGTATCTGGCCATGTCCTCTG-3';[22] AgRP primers, 5'-TTGTGTTCTGCTGTTGGCACT-3' and 5'-AGCAAAAGGCATTGAAGAAGC-3';[22] and β-actin primers, 5'-ATGGGTCAGAAGGACTCCTACG-3' and 5'-AGTGGTACGACCAGAGGCATAC-3'.[23]

4.11. Determination of serotonin (5-HT) release from ileum

Male ddY mice about 35–36 g were bled to death by severing both arteries under ether anesthesia and the ileum was removed and cut into segments (about 10 mm, wet weight 0.03–0.05 g). Ileum segments were placed into 1 mL of modified Krebs's solution (NaCl 120.0; KCl 5.0; $CaCl_2$ 2.5; $MgSO_4$ 1.0; NaH_2PO_4 1.0; $NaHCO_3$ 25.0; glucose 11.0 mM) with or without a test sample, maintained at 37 °C. It was aerated with a 95% O_2–5% CO_2 gas mixture and kept pH 7.4. After 20 min, 100 μL of the incubation buffer was added to the 15 μL of 1 M CH_3COOH to adjust the pH to ca. 3.0 and filtered (0.45 μm pore size filter). These samples were stored at −80 °C until determination.

After incubation with a test sample, tissue was moved into 1 mL of a new modified Krebs's solution and homogenized by TissueLyser (Qiagen). The tissue homogenate (100 μL) was mixed with 100 μL of 0.5 M $HClO_3$, and centrifuged (15,000g, 4 °C, 15 min). The supernatant (100 μL) was mixed with 20 μL of 1 M CH_3COONa to adjust the pH to ca. 3.0 and filtered (0.45 μm pore size filter). These samples were stored at −80 °C until determination.

5-HT levels in the solutions were determined by high performance liquid chromatography (HPLC) [column: Eicompak CA-5 ODS (2.1 mm, i.d. × 150 mm), solvent: 0.1 M phosphate buffer (pH 6.0)/methanol 80:20 (v/v), flow rate: 0.23 mL/min] with an electrochemical detector (ECD) (ECD-300, Eicom, Kyoto, Japan), and 10 ng/mL of isoproterenol (w/v) was used as an internal standard.

4.12. Statistical analyses

Values are expressed as the mean ± SEM. A one-way analysis of variance followed by Dunnett's test was used for statistical analyses. Probability (p) values of less than 0.05 were considered to be significant.

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