Original paper

Hypolipidemic Property of a New Fermented Tea Made with Third Crop Green Tea (*Camellia sinensis*) Leaves and Unripe Satsuma Mandarin (*Citrus unshiu*) Fruits

Hisayuki NAKAYAMA^{1,2*}, Naho YUITO³, Yuji MIYATA², Kei TAMAYA⁴, Takashi TANAKA⁵, Yoshinori SAITO⁵, Toshiro MATSUI⁶, Sadayuki ARAMAKI², Yasuo NAGATA^{3,7}, Shizuka TAMARU³ and Kazunari TANAKA^{1,3}

¹Graduate School of Human Health Science, University of Nagasaki, 1-1-1 Manabino, Nagayo-cho, Nishisonogi-gun, Nagasaki 851-2195, Japan

²Nagasaki Agricultural and Forestry Technical Development Center, 3118 Kaizu-cho, Isahaya, Nagasaki 859-0063, Japan

³Department of Nutrition, University of Nagasaki, 1-1-1 Manabino, Nagayo-cho, Nishisonogi-gun, Nagasaki 851-2195, Japan

⁴Industrial Technology Center of Nagasaki, 2-1303-8 Ikeda, Ohmura, Nagasaki 856-0026, Japan

⁵Graduate School of Biochemical Science, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

⁶Faculty of Agriculture, Graduate School of Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

⁷Center for Industry, University and Government Cooperation, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

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We manufactured a new fermented tea by tea-rolling processing of third crop green tea (*Camellia sinensis*) leaves and unripe satsuma mandarin (*Citrus unshiu*) fruits, and investigated the effects of feeding the tea extract on serum and liver lipid concentrations in rats. The tea extract contained narirutin and hesperidin from unripe satsuma mandarin fruits, catechins from green tea leaves, and black tea polyphenols produced by oxidation of catechins. The fermented tea extract inhibited pancreatic lipase activity *in vitro*. When rats were fed diets supplemented with the freeze-dried tea extract (0.50% or 0.75%) for 4 weeks, hepatic triglyceride and cholesterol concentrations were reduced in a dose-dependent manner, and the reductions were significant in rats fed diet composed of 0.75% tea extract compared to those fed the control diet. These results suggest that the tea produced by mixing third crop green tea leaves and unripe satsuma mandarin fruits has a hypolipidemic property.

Keywords: fermented tea, third crop green tea leaves, unripe satsuma mandarin fruits, polyphenols, hypolipidemic property

Abbreviations

CPT; carnitine palmitoyltransferase, CYP7A1; cholesterol 7 α -hydroxylase, EC; epicatechin, ECG; epicatechin-3-*O*-gallate, EGCG; epigallocatechin, EGCG; epigallocatechin-3-*O*-gallate, FAS; fatty acid synthase, GT; green tea extract, G6PDH; glucose 6-phosphate dehydrogenase, HMG-CoA; 3-hydroxy-3-methylglutaryl CoA, HPLC; high performance liquid chromatography, mixUM-GT; mixed fermented tea extract, PAP; phosphatidic acid phosphohydrolase, PCR; polymerase chain reaction, UM; unripe satsuma mandarin fruit extract

*To whom correspondence should be addressed.

E-mail: hnakayama1206@pref.nagasaki.lg.jp

Introduction

Tea is commonly consumed worldwide, and green tea (*Camellia sinensis*) is the most popular beverage in Asian countries. Green tea is recognized to have beneficial effects on human health, which are attributed to polyphenolic compounds known as catechins: epicatechin (EC), epicatechin-3-*O*-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-*O*-gallate (EGCG) (Lambert *et al.*, 2005), and are evidenced by various functions such as antioxidant, anticancer, hypoglycemic, and hypolipidemic properties (Crespy and Williamson, 2004).

Green tea leaves are harvested 3 – 4 times per year at a tea plantation. After the first harvest, which takes place from the middle of April to the beginning of May, the commercial value of tea leaves gradually diminishes because of the decreasing amount of amino acid analogues such as theanine, which are associated with good taste, and the increasing content of catechins, which impart a bitter taste. Levels of theanine and catechins in the third crop green tea leaves are approximately 10% and 130% of those in the first crop green tea leaves, respectively (Kanbe *et al.*, 2005). Hence, the taste of third crop green tea leaves harvested in the summer season is inferior to first crop green tea leaves; thus, they are not effectively utilized and are in part discarded.

Tanaka *et al.* (2002a) observed that unripe satsuma mandarin (*Citrus unshiu*) fruits accelerate tea catechin oxidation, inducing the formation of black tea polyphenols, i.e., theaflavins, theasinensins, and thearubigins as principal components of fermented teas such as black tea. Matsumoto *et al.* (1998) showed that black tea polyphenols exert hypocholesterolemic and hypotriglyceridemic effects in cholesterol-fed rats. Keemun black tea extract has been observed to reduce body weight in rats (Du *et al.*, 2005). Miyata *et al.* (2011) demonstrated that black tea polyphenols, especially theaflavins and theasinensins, exert cholesterol-lowering activity through the stimulation of fecal steroid excretion in rats. Furthermore, it has been reported that black tea, rather than green tea, suppresses cholesterol synthesis in cultured rat hepatoma cells (Singh *et al.*, 2009). Thus, black tea polyphenols generated by polymerization of catechins appear to improve lipid metabolism.

Satsuma mandarin belongs to the *Rutaceae* family, which is popular for its fragrance and juiciness. Satsuma mandarin fruits contain special types of flavonoids known as flavanone glycosides. These glycosides include hesperidin, which exhibits biological and pharmacological properties such as reduction of capillary fragility and hypotriglyceridemic activity (Bok *et al.*, 1999; Garg *et al.*, 2001; Jung *et al.*, 2006). The amount of hesperidin is four times greater in unripe satsuma mandarin fruits harvested in July than in those harvested in December (Ando *et al.*, 2011). However, to improve the quality of the remaining fruits, large amounts of unripe fruits are thinned out and discarded as waste.

Miyata *et al.* (2009) developed a new fermented tea by kneading third crop green tea leaves and loquat (*Eriobotrya*

japonica) leaves. Loquat leaves potentially accelerated the oxidation of catechins contained in third crop green tea leaves, consequently generating black tea polyphenols during fermentation (Tanaka *et al.*, 2002a). The new fermented tea exhibited hypotriglyceridemic and antiobesity effects (Tanaka *et al.*, 2010). Thus, if third crop green tea leaves and unripe satsuma mandarin fruits were mixed and fermented, the black tea polyphenols and hesperidin contained in the product could be expected to exert human health benefits.

In the present study, we investigated the effects of feeding a new fermented tea made with third crop green tea leaves and unripe satsuma mandarin fruits on serum and liver lipid concentrations in Sprague-Dawley (SD) rats.

Materials and Methods

Materials Third crop green tea leaves and unripe satsuma mandarin fruits were provided by the Nagasaki Agricultural and Forestry Technical Development Center (Nagasaki, Japan), and were harvested in August and July of 2011, respectively. Fresh tea leaves were dried by blowing air (70°C) for 20 min in a primary tea-rolling dryer (60k-type; Kawasaki Co., Ltd., Shimada, Japan). The temperature of the leaves did not exceed 40°C during this process. Then, the leaves and sliced unripe satsuma mandarin fruits were mixed at a ratio of 3:1 and kneaded by a tea roller (60k-type; Kawasaki Co.) at room temperature for 20 min. Finally, the mixture was heated at 110°C in a tea dryer (120k-2 type; Kawasaki Co.) for 30 min to terminate enzymatic oxidation. The mixture (20 g) was added to 1 L of hot water and stirred for 10 min. The extract was filtered, and concentrated using a rotary evaporator. The concentrate was lyophilized by freeze drying (FD-550R; Tokyo Rikakikai, Tokyo, Japan), and powdered to generate the mixed fermented tea extract (mixUM-GT). To prepare the green tea extract (GT), third crop green tea leaves were steamed to inactivate polyphenol oxidase, then kneaded using a tea roller and dried using a tea dryer. The water content of green tea leaves was less than 5%. Finally, green tea was extracted using the same method as for mixUM-GT. Sliced unripe satsuma mandarin fruit (20 g) was added to 1 L of hot water, and the extract was subsequently filtered, lyophilized, and powdered to generate the unripe satsuma mandarin fruit extract (UM).

Polyphenols contained in UM, GT, and mixUM-GT were extracted with 60% ethanol, and their concentrations were then analyzed using HPLC. Caffeine and gallic acid were purchased from Nacalai Tesque (Kyoto, Japan), and catechin and hesperidin were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Catechin-3-*O*-gallate, gallocatechin-3-*O*-gallate, and naringin were estimated by using standard curves of ECG, EGCG, and naringin (Tokyo Chemical Industry Co., Ltd.), respectively. EC, ECG, EGC, and EGCG were isolated from commercial green tea according to Nonaka *et al.* (1983) and recrystallized from H₂O. Gallocatechin (Matsuo *et al.*, 2008), theaflavins (Tanaka *et al.*, 2002a), theasinensins A and B (Tanaka *et al.*, 2003; Shii *et al.*,

2011), and polymeric polyphenols (Tanaka *et al.*, 2009) were prepared as described elsewhere. Analytical HPLC conditions were as follows: Cosmosil 5C₁₈-AR II (Nacalai Tesque) column (4.6 mm i.d. × 250 mm) with gradient elution from 4 to 30% (39 min) and 30 to 75% (15 min) of CH₃CN in 50 mM H₃PO₄; flow rate, 0.8 mL/min; detection, photodiode array detector (MD-910; Jasco Co., Ltd., Tokyo).

Measurement of pancreatic lipase activity The pancreatic lipase activity was measured using 4-methylumbelliferyl oleate (4-MU oleate) as a substrate (Kurihara *et al.*, 2003). Twenty-five microliters of UM, GT, or mixUM-GT solution dissolved in water and 50 μ L of 0.1 mM 4-MU solution dissolved in a buffer consisting of 13 mM Tris-HCl, 150 mM NaCl, and 1.3 mM CaCl₂ (pH 8.0) were mixed in the well of a microtiter plate, and then 25 μ L of the lipase solution (50 U/mL) in the above buffer was added to start the enzyme reaction. After incubation at 25°C for 30 min, 0.1 mL of 0.1 M sodium citrate (pH 4.2) was added to stop the reaction. The amount of 4-methylumbelliferone released by the lipase was measured with a fluorometric microplate reader (Varioskan version 2.2; Thermo Fisher Scientific, Kanagawa, Japan) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The IC₅₀ of the test sample was obtained from the least-squares regression line of the logarithmic plots of the sample concentration (log) versus the pancreatic lipase activity (%).

Effects of UM, GT, or mixUM-GT on serum and liver lipid concentrations in rats (Experiment I) Four-week-old male SD rats were purchased from Clea Japan (Tokyo). Rats were individually housed in stainless-steel cages under a controlled atmosphere (temperature, 22 ± 1°C; humidity, 55 ± 5%; light cycle, 08:00 – 20:00), fed a commercial pellet diet (CE-2; Clea Japan), and allowed free access to water for one week. All animal studies were carried out under the Guidelines for Animal Experiments of University of Nagasaki (Nagasaki), and Law No. 105 and Notification No. 6 of the government of Japan. Rats were assigned to 4 groups of 6 – 7 animals each. The control diet was prepared according to the modified AIN-93G composition. MixUM-GT was added to the control diet at a level of 0.75% at the expense of cornstarch. In our preliminary experiment, food intake of rats tended to decrease when mixUM-GT was added at the level of more than 1.0% to diets, thus the upper limit of mixUM-GT addition was set at 0.75%. In the manufacturing of mixUM-GT, since green tea leaves and unripe satsuma mandarin fruits were used at a 3:1 ratio, UM and GT were added to the control diet at a level of 0.19% and 0.56%, respectively, which was equivalent to each level used to prepare the 0.75% mixUM-GT diet.

The rats were given free access to the diets and water for 4 weeks. Food intake and body weight were recorded daily. After fasting for 6 h, the rats were sacrificed by decapitation. Blood was collected, and perirenal, epididymal, and mesenteric white adipose tissue, brown adipose tissue, and liver were immediately excised and

weighed. The concentrations of serum triglyceride, cholesterol, and phospholipid were enzymatically assayed using commercial kits (Triglyceride E-test, Cholesterol E-test, and Phospholipid C-Test, respectively; Wako Pure Chemical Industries, Osaka, Japan). Liver lipids were extracted by the method of Folch *et al.* (1957) and then triglyceride, cholesterol, and phospholipid concentrations were enzymatically measured as described above.

Effect of mixUM-GT dietary level on serum and liver lipid concentrations in rats (Experiment II) Rats were given a modified AIN-93G diet containing mixUM-GT at a level of 0.50% or 0.75% for 4 weeks. In this experiment, to elucidate the minimum mixUM-GT content required to exert hypolipidemic activity, the dosage of 0.50% was also used. Rats were sacrificed by decapitation after 6 h fasting, and analyses were carried out as outlined in Experiment I. Enzyme activities related to lipogenesis and lipolysis in liver and brown adipose tissue were also measured. In addition, the gene expression of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase and cholesterol 7 α -hydroxylase (CYP7A1) in liver were determined by real-time PCR.

Preparation of hepatic subcellular fractions The liver was excised, immediately frozen using liquid nitrogen, and stored at –80°C until analysis. The liver was homogenized in 6 volumes of a 0.25 M sucrose solution containing 1 mM of EDTA in 10 mM of Tris-HCl buffer (pH 7.4). After separation of the nuclear fraction, the supernatant was centrifuged at 100,000 × *g* for 60 min to precipitate microsomes, and the remaining supernatant was used as the cytosolic fraction. Mitochondrial and microsomal pellets were resuspended in a 0.25 M sucrose solution containing 1 mM of EDTA in 10 mM of Tris-HCl buffer (pH 7.4).

Measurement of enzyme activities in liver and brown adipose tissue Activities of cytosolic fatty acid synthase (FAS) (Kelly *et al.*, 1986), glucose 6-phosphate dehydrogenase (G6PDH) (Kelly and Kletzien, 1984), malic enzyme (Ochoa, 1955), microsomal phosphatidic acid phosphohydrolase (PAP) (Walton and Possmayer, 1985), and mitochondrial carnitine palmitoyltransferase (CPT) (Markwell *et al.*, 1973) in liver were determined. CPT activity in brown adipose tissue homogenate was also measured. Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

RNA isolation and quantitative real-time PCR Total RNA was isolated from 100- μ g liver samples using an RNA-iso Plus (Takara Bio Inc., Shiga, Japan). The RNA samples were converted into complementary DNA (cDNA) using PrimeScript RT Master Mix (Takara Bio Inc.). Relative levels of HMG-CoA reductase and CYP7A1 mRNA were quantified using the THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) and an ABI 7300 instrument (Applied Biosystems Inc., Tokyo, Japan). The primers were designed in reference to the GenBank database using Primer Express software (Applied Biosystems Inc.). The 5' and 3' primers for HMG-CoA reductase were 5'-tgcagagaaagggtcgaagt-3' and 5'-cgtctccatgagggtttcca-3', respectively. The 5' and 3' primers for

Table 1. Polyphenolic concentrations in unripe satsuma mandarin fruit extract, green tea extract, and mixed fermented tea extract

Polyphenolic Compounds	UM	GT		MixUM-GT	
	(mg/100 g UM diet*)	(mg/100 g GT diet*)		(mg/100 g MixUM-GT*)	
Caffeine	N.D.	25.5 ±	2.3	47.4 ±	1.6
Gallic acid	N.D.	1.46 ±	0.11	6.55 ±	0.15
Catechin	N.D.	6.08 ±	0.60	N.D.	
Catechin-3- <i>O</i> -gallate	N.D.	5.04 ±	0.43	N.D.	
Gallocatechin	N.D.	34.1 ±	2.8	N.D.	
Gallocatechin-3- <i>O</i> -gallate	N.D.	26.8 ±	2.3	2.56 ±	0.04
EC	N.D.	8.30 ±	0.59	7.19 ±	0.14
ECG	N.D.	6.33 ±	0.53	13.7 ±	0.3
EGC	N.D.	28.4 ±	2.3	15.9 ±	0.4
EGCG	N.D.	32.7 ±	2.8	48.4 ±	1.1
Theasinensin A	N.D.	N.D.		5.94 ±	0.12
Theasinensin B	N.D.	N.D.		8.45 ±	0.41
Polymeric polyphenols	N.D.	N.D.		31.9 ±	1.4
Theaflavins	N.D.	N.D.		5.10 ±	0.09
Narirutin	1.52 ± 0.11	N.D.		10.6 ±	0.2
Hesperidin	1.03 ± 0.06	N.D.		8.33 ±	0.20

*A hundred grams of UM diet, GT diet, or mixUM-GT diet contained 0.19 g UM, 0.56 g GT, or 0.75 g MixUM-GT, respectively. Values are means ± SEM (n = 3).

N.D., Not detected.

UM, Unripe satsuma mandarin fruit extract.

GT, Green tea extract.

MixUM-GT, Mixed fermented tea extract.

EC, Epicatechin.

ECG, Epicatechin-3-*O*-gallate.

EGC, Epigallocatechin.

EGCG, Epigallocatechin-3-*O*-gallate.

CYP7A1 were 5'-tacttctcggaaggcatttg-3' and 5'-atctccctggagggttttg-3', respectively. The level of 36B4 mRNA was measured and served as the reference gene. The 5' and 3' primers for 36B4 were 5'-cgaaggcttgaatcctaacgaa-3' and 5'-gttgacctgcagctgtttgc-3', respectively. The amplification reactions were performed under the following PCR conditions: initial denaturation at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s, and extension at 60°C for 34 s. Melting curves were analyzed to ensure that fluorescence signals solely reflected specific amplicons.

Statistical analysis All data were expressed as means ± SEM. Statistical analyses were performed using the Statistical Package for Social Science (SPSS) software (IBM Japan, Ltd., Tokyo, Japan). Significant differences among groups were determined by one-way ANOVA, followed by the Tukey-Kramer test. Values were considered significantly different at a *p* value of <0.05.

Results

Polyphenolic concentrations contained in the UM, GT, and mixUM-GT diets are summarized in Table 1. Narirutin and hesperidin were detected in UM. GT contained caffeine, gallic acid, and catechins. MixUM-GT contained caffeine, gallic acid, catechins, black tea polyphenols (theasinensin A, theasinensin B,

polymeric polyphenols, and theaflavins), narirutin, and hesperidin. The phenolic composition of mixUM-GT differed somewhat from that of GT. Catechin, catechin-3-*O*-gallate, and gallocatechin were not detected in mixUM-GT, and the concentrations of gallocatechin-3-*O*-gallate and EGC were lower in mixUM-GT than in GT. The concentration of caffeine was 1.9 times higher in mixUM-GT than in GT. Theasinensins (theasinensin A and theasinensin B), polymeric polyphenols, and theaflavins were detected only in mixUM-GT. The levels of narirutin and hesperidin detected in mixUM-GT were 7.0 and 8.1 times higher than those in UM, respectively.

Pancreatic lipase activity in vitro Pancreatic lipase IC₅₀s were determined using 4-MU oleate as a substrate. The inhibitory activities of GT (IC₅₀ = 76.2 ± 23.4 µg/mL, n = 3) and mixUM-GT (IC₅₀ = 64.6 ± 9.9 µg/mL, n = 3) were similar, and were much greater than that of UM (IC₅₀ = 637 ± 6 µg/mL, n = 3).

Effects of UM, GT, or mixUM-GT on serum and liver lipid concentrations in rats (Experiment I) Food intake did not significantly differ among all groups. The feeding of GT and mixUM-GT diets tended to decrease the final body weight (Table 2), which was significantly lower in the GT group than in the UM group. Liver weight was comparable irrespective of the diet.

Table 2. Effects of dietary unripe satsuma mandarin fruit extract, green tea extract, and mixed fermented tea extract on growth parameters in rats

	Control	UM	GT	MixUM-GT
Initial body weight (g)	153 ± 2	154 ± 2	154 ± 2	154 ± 2
Final body weight (g)	420 ± 11 ^{ab}	432 ± 13 ^a	390 ± 5 ^b	399 ± 9 ^{ab}
Food intake (g/day)	26.1 ± 0.8	27.8 ± 1.0	25.1 ± 0.6	25.4 ± 0.8
Liver weight (g/100 g body weight)	3.64 ± 0.11	3.78 ± 0.17	3.64 ± 0.13	3.58 ± 0.11
White adipose tissue weights (g/100 g body weight)				
Perirenal	2.16 ± 0.22	2.43 ± 0.17	1.80 ± 0.08	1.80 ± 0.21
Epididymal	1.39 ± 0.09	1.45 ± 0.12	1.14 ± 0.05	1.15 ± 0.06
Mesenteric	1.35 ± 0.11 ^{ab}	1.63 ± 0.16 ^a	1.15 ± 0.06 ^b	1.08 ± 0.06 ^b
Perirenal + Epididymal + Mesenteric	4.91 ± 0.38 ^{ab}	5.51 ± 0.43 ^a	4.09 ± 0.14 ^b	4.03 ± 0.31 ^b
Brown adipose tissue weight (g/100 g body weight)	0.126 ± 0.005	0.127 ± 0.008	0.116 ± 0.008	0.125 ± 0.011

Values are means ± SEM for 6 – 7 rats per group.

^{ab}Different superscript letters show significant differences at $p < 0.05$.

UM, Unripe satsuma mandarin fruit extract.

GT, Green tea extract.

MixUM-GT, Mixed fermented tea extract.

Table 3. Effects of dietary unripe satsuma mandarin fruit extract, green tea extract, and mixed fermented tea extract on serum and liver lipid levels in rats

	Control	UM	GT	MixUM-GT
Serum (mg/100 ml)				
Triglyceride	152 ± 23	167 ± 24	111 ± 13	114 ± 16
Cholesterol	84.3 ± 5.3 ^{ab}	102.9 ± 5.5 ^a	81.6 ± 4.0 ^b	90.9 ± 4.3 ^{ab}
Phospholipid	187 ± 8 ^{ab}	215 ± 10 ^a	169 ± 4 ^b	190 ± 7 ^{ab}
Liver (mg/g liver)				
Triglyceride	41.5 ± 2.8 ^{ab}	46.1 ± 3.4 ^a	31.4 ± 1.6 ^{bc}	30.8 ± 2.4 ^{cd}
Cholesterol	4.24 ± 0.25 ^b	5.32 ± 0.22 ^a	3.68 ± 0.32 ^b	3.68 ± 0.19 ^b
Phospholipid	17.9 ± 1.0	18.2 ± 1.2	19.3 ± 1.0	17.7 ± 0.8

Values are means ± SEM for 6 – 7 rats per group.

^{abcd}Different superscript letters show significant differences at $p < 0.05$.

UM, Unripe satsuma mandarin fruit extract.

GT, Green tea extract.

MixUM-GT, Mixed fermented tea extract.

Perirenal, epididymal, mesenteric, and the sum of white adipose tissue weights tended to increase in the UM group and decrease in the GT and mixUM-GT groups compared to the control, and the values of mesenteric and total white adipose tissue weights in the GT and mixUM-GT groups were significantly lower than those in the UM group. Brown adipose tissue weight was the same among the groups.

The GT and mixUM-GT diets slightly, but not significantly, lowered the serum triglyceride concentration compared to the control and UM diets (Table 3). Although serum cholesterol and phospholipid concentrations did not differ among the control, GT, and mixUM-GT groups, the GT group value was significantly lower than that of the UM group. Hepatic triglyceride concentration in the mixUM-GT group was the lowest among the four groups, and differed significantly compared to the control and UM groups. However, the GT diet did not significantly lower hepatic triglyceride level compared to the control. Hepatic cholesterol concentration was significantly lower in the control, GT, and mixUM-GT groups than in the UM group; however, significant

differences were not observed among the control, GT, and mixUM-GT groups. Hepatic phospholipid concentration was not affected by the diets.

Effects of mixUM-GT dietary level on serum and liver lipid concentrations in rats (Experiment II) No effect of diets was seen in food intake; however, the 0.50% and 0.75% mixUM-GT diets significantly reduced the final body weight compared to the control (Table 4). Perirenal, epididymal, mesenteric, and total white adipose tissue weights tended to be lower, although not significantly so, in the rats fed mixUM-GT diets than those fed the control diet. No differences were observed in liver and brown adipose tissue weights among the groups.

The serum triglyceride, cholesterol, and phospholipid concentrations tended to be reduced by mixUM-GT diets compared to the control; however, these were non-significant differences (Table 5). The hepatic triglyceride concentration dose-dependently decreased with mixUM-GT diet feeding; the 0.75% addition significantly decreased levels by 59% compared to the control. The hepatic cholesterol concentration also decreased in a dose-

Table 4. Effects of dietary mixed fermented tea extract on growth parameters in rats

	Control	MixUM-GT	
		0.50%	0.75%
Initial body weight (g)	149 ± 3	149 ± 3	149 ± 2
Final body weight (g)	389 ± 7 ^a	340 ± 9 ^b	357 ± 10 ^b
Food intake (g/day)	22.0 ± 0.6	20.2 ± 0.6	21.3 ± 0.7
Liver weight (g/100 g body weight)	3.77 ± 0.12	3.90 ± 0.15	3.81 ± 0.08
White adipose tissue weights (g/100 g body weight)			
Perirenal	1.67 ± 0.21	1.19 ± 0.08	1.20 ± 0.21
Epididymal	1.34 ± 0.12	1.18 ± 0.06	1.15 ± 0.07
Mesenteric	1.04 ± 0.05	0.97 ± 0.05	0.95 ± 0.12
Perirenal + Epididymal + Mesenteric	4.05 ± 0.29	3.34 ± 0.15	3.30 ± 0.39
Brown adipose tissue weight (g/100 g body weight)	0.14 ± 0.02	0.15 ± 0.01	0.16 ± 0.02

Values are means ± SEM for 6 rats per group.

^{ab}Different superscript letters show significant differences at $p < 0.05$.

MixUM-GT, Mixed fermented tea extract.

Table 5. Effects of dietary mixed fermented tea extract on serum and liver lipid levels in rats

	Control	MixUM-GT	
		0.50%	0.75%
Serum (mg/100 ml)			
Triglyceride	267 ± 22	207 ± 18	230 ± 18
Cholesterol	94.2 ± 5.4	79.2 ± 4.2	83.9 ± 7.7
Phospholipid	145 ± 7	123 ± 6	131 ± 8
Liver (mg/g liver)			
Triglyceride	33.5 ± 6.9 ^a	20.1 ± 2.6 ^{ab}	13.8 ± 2.2 ^b
Cholesterol	4.14 ± 0.50 ^a	3.01 ± 0.19 ^{ab}	2.49 ± 0.13 ^b
Phospholipid	15.5 ± 0.4	14.1 ± 0.8	13.3 ± 0.4

Values are means ± SEM for 6 rats per group.

^{ab}Different superscript letters show significant differences at $p < 0.05$.

MixUM-GT, Mixed fermented tea extract.

dependent manner with the mixUM-GT diet; a significant reduction compared to the control was observed in the 0.75% mixUM-GT group. The hepatic phospholipid concentration was identical among the groups.

FAS activity showed a dose-dependent decreasing trend with mixUM-GT diet intake; however, a significant difference compared to the control was not observed (Table 6). Supplementation with mixUM-GT did not affect the activities of hepatic G6PDH, malic enzyme, PAP, CPT, and brown adipose tissue CPT, irrespective of the mixUM-GT level.

When mixUM-GT was added to the diets at a level greater than 0.50%, hepatic gene expression of HMG-CoA reductase doubled, and that of CYP7A1 increased 3–4 times (Table 6); however, the observed increases were not significant.

Discussion

To elucidate the function of a new fermented tea produced by mixing third crop green tea leaves and unripe satsuma mandarin fruits, which are currently not effectively utilized, we investigated the effects of feeding the fermented tea extract on serum and liver

lipid concentrations in rats.

The present study showed that the mixUM-GT diet decreased hepatic triglyceride concentrations in a dose-dependent manner (Tables 3 and 5). The reduction of liver triglyceride level is known to be induced through the suppression of hepatic lipogenesis, the acceleration of hepatic lipolysis, and/or the inhibition of intestinal fat absorption. The mixUM-GT diet reduced hepatic FAS activity in a dose-dependent manner (Table 6), although not significantly so. Although the suppression of hepatic FAS activity may contribute to the hepatic hypotriglyceridemic property of mixUM-GT, this activity does not appear to be strong. Han *et al.* (2001) and Ikeda *et al.* (2005) pointed out that the suppression of dietary fat absorption in the intestine by inhibition of pancreatic lipase activity decreases the hepatic triglyceride level. MixUM-GT, as well as GT, showed pancreatic lipase inhibitory activity *in vitro*. It is possible that the inhibition of pancreatic lipase activity by dietary mixUM-GT induced the suppression of intestinal fat absorption. Hence, the reduction of hepatic triglyceride concentration in rats fed mixUM-GT might be additively induced by the suppression of liver fatty acid synthesis and the inhibition of fat absorption from

Table 6. Effects of dietary mixed fermented tea extract on lipogenic and lipolytic enzyme activities of liver and brown adipose tissue, and cholesterol synthesis and catabolism gene expression in liver of rats

	Control	MixUM-GT	
		0.50%	0.75%
Enzyme activities		nmol/min/mg protein	
Liver			
Fatty acid synthase (cytosol)	3.51 ± 0.60	3.01 ± 0.93	2.83 ± 0.47
Glucose 6-phosphate dehydrogenase (cytosol)	39.3 ± 5.8	42.6 ± 8.9	46.2 ± 7.2
Malic enzyme (cytosol)	36.9 ± 5.7	48.5 ± 9.4	42.2 ± 5.1
Phosphatidic acid phosphohydrolase (microsome)	38.5 ± 3.0	47.6 ± 7.6	35.8 ± 3.0
Carnitine palmitoyltransferase (mitochondria)	4.51 ± 0.48	5.24 ± 0.63	4.73 ± 0.97
Brown adipose tissue			
Carnitine palmitoyltransferase (homogenate)	9.94 ± 0.37	11.85 ± 1.50	8.79 ± 0.96
Gene expressions		relative expression	
HMG-CoA reductase	1.00 ± 0.21	1.95 ± 0.64	1.99 ± 0.51
CYP7A1	1.00 ± 0.20	4.40 ± 1.68	2.95 ± 0.52

Values are means ± SEM for 6 rats per group.

MixUM-GT, Mixed fermented tea extract.

HMG-CoA reductase, 3-Hydroxy-3-methylglutaryl CoA reductase.

CYP7A1, Cholesterol 7 α hydroxylase.

the small intestine.

Supplementation with mixUM-GT exhibited the most potent liver triglyceride-lowering activity among the three extracts (Table 3). GT also decreased hepatic triglyceride level compared to the control diet. However, the difference in hepatic hypotriglyceridemic effect between GT and mixUM-GT was not very large. In our previous study, an extract of third crop green tea leaves was found to exert hypotriglyceridemic action through the stimulation of lipolysis (Tanaka *et al.*, 2010). In this study, mixUM-GT did not enhance CPT activities in either liver or brown adipose tissue (Table 6), even though green tea leaves were used as a component of mixUM-GT. Therefore, the mechanism of action of GT and mixUM-GT on triglyceride metabolism appears to differ. This result strongly suggests that the hypotriglyceridemic effect of mixUM-GT is due to the mixUM-GT itself and not to the third crop green tea leaves.

MixUM-GT exhibited a dose-dependent reduction of hepatic cholesterol concentration in experiment II (Table 5); the 0.75% supplementation level showed a significant effect. Hepatic CYP7A1 gene expression in rats fed mixUM-GT diet at a greater than 0.50% supplementation level was 3–4 times higher, although not significantly so, compared to the control (Table 6). Enhanced CYP7A1 activity is known to induce a reduction in liver cholesterol concentration (Boone *et al.*, 2011). Therefore, the reduction of hepatic cholesterol level by dietary mixUM-GT is thought to be in part due to the acceleration of liver cholesterol catabolism. Acceleration of fecal steroid excretion (Miyata *et al.*, 2011) and inhibition of synthesis and/or secretion of lipoproteins and apolipoproteins (Temel *et al.*, 2007) strongly induce cholesterol-lowering activity. In the future we intend to examine

these parameters. However, a significant reduction of hepatic cholesterol concentration was not observed in experiment I. Moreover, mixUM-GT did not lower the serum cholesterol level. Therefore, the hypocholesterolemic effect of mixUM-GT may not necessarily be potent.

MixUM-GT contained narirutin and hesperidin from unripe satsuma mandarin fruits, and caffeine and catechins from green tea leaves (Table 1). The amount of caffeine was 1.9 times higher in the mixUM-GT diet than in the GT diet. Li *et al.* (2008) determined that the concentration of caffeine contained in tea leaves was increased during fermentation. Thus, the higher content of caffeine in mixUM-GT compared to GT is proposed to be induced by fermentation. It is reported that catechin and caffeine in green tea exerted synergistic effects on enhancing fat expenditure in obese mice (Zheng *et al.*, 2004), and several studies showed that caffeine had lipid-lowering activity (Saito *et al.*, 1974; Kobayashi-Hattori *et al.*, 2005; Quan *et al.*, 2013). Shimoda *et al.* (2006) suggested that caffeine was the compound contributing to the suppression of fat absorption in mice, and this effect was observed when 0.05% caffeine was added to the diet. Because the caffeine content was approximate 0.05% in mixUM-GT, the hypolipidemic activity of mixUM-GT may be in part attributed to caffeine.

Black tea polyphenols were detected in the mixUM-GT extract. Since black tea polyphenols are produced by the oxidation of catechins, it is likely that mixUM-GT was fully fermented by the kneading process alone, without the oxidation process. Black tea polyphenols were reported to induce a hypotriglyceridemic effect (Matsumoto *et al.*, 1998). We also reported that a fermented tea rich in black tea polyphenols, e.g., theaflavins, theasinensins, and

thearubigins, showed hypotriglyceridemic and antiobesity properties (Tanaka *et al.*, 2010). Recently, Miyata *et al.* (2013) found that theasinensins exhibited hepatic hypotriglyceridemic effects in rats. Kusano *et al.* (2008) reported the pancreatic lipase inhibitory activity of theaflavins. Moreover, Nakai *et al.* (2005) observed that theasinensin A and theasinensin B, as well as theaflavins, strongly inhibited pancreatic lipase activity. We speculate that the liver triglyceride-lowering effect of mixUM-GT is in part induced through the suppression of intestinal triglyceride absorption by caffeine and black tea polyphenols such as theaflavins and theasinensins.

Fujita *et al.* (2008) showed that unripe satsuma mandarin fruits contain high levels of narirutin and hesperidin. Citrus flavonoids, especially hesperidin, are also promising in terms of their hypotriglyceridemic function (Bok *et al.*, 1999; Garg *et al.*, 2001; Jung *et al.*, 2006). Even though UM contains hesperidin, it did not lower serum and liver triglyceride levels (Table 3). Hesperidin at 20 mg/100 g diet exhibited an effective hypotriglyceridemic action in rats (Kim *et al.*, 2003). In this study, we added UM powder to the control diet at a level of 0.19%, which gives only 1 mg of hesperidin per 100 g diet (Table 1). Therefore, it is likely that the hesperidin content in the UM diet was too low to exert a hypotriglyceridemic effect. Although the amount of unripe satsuma mandarin fruits used to produce UM and mixUM-GT was the same, the levels of hesperidin and narirutin detected in mixUM-GT were 7–8 times greater than those in UM (Table 1). Notably, hesperidin and narirutin are insoluble in water and alcohol (Okamura *et al.*, 2000; Yamada *et al.*, 2006). In the present study, because unripe fruits were directly extracted with hot water to produce UM, the amount of hesperidin and narirutin extracted from unripe satsuma mandarin fruits was estimated to be slight. Tanaka *et al.* (1997 and 2002b) demonstrated that the coexistence of catechins and/or black tea polyphenols with hydrophobic substances such as hesperidin remarkably increased their solubility in water. In fact, we confirmed the increase in hesperidin solubility in heated water by the coexistence of catechins and black tea polyphenols in a fermented tea product made from third crop green tea leaves and unripe satsuma mandarin fruits (Nakayama *et al.*, 2014). As shown in Table 1, hesperidin contained in mixUM-GT was approximately 10 mg; however, the lower concentration limit of hesperidin that induces a hypotriglyceridemic effect is obscure. Therefore, we suggest that hesperidin was eluted during the manufacturing of mixUM-GT, contributing in part to the reduction of liver triglyceride level.

It has been demonstrated that catechins, black tea polyphenols, and hesperidin exhibit cholesterol-lowering activity (Ikeda *et al.*, 1992; Matsumoto *et al.*, 1998; Bok *et al.*, 1999; Chan *et al.*, 1999; Maron *et al.*, 2003). The hypocholesterolemic effect of mixUM-GT may be induced by these components. Lee *et al.* (2008) pointed out that ECG and EGCG reduced liver cholesterol concentrations through enhanced CYP7A1 mRNA expression in HepG2 cells.

Miyata *et al.* (2011) showed that black tea polyphenols such as theaflavins, theasinensins, and thearubigins induced cholesterol-lowering activity in the liver through the stimulation of fecal steroid excretion in rats. Hesperidin was reported to exert a hypocholesterolemic effect through the inhibition of apoB secretion, the suppression of acyl CoA cholesterol acyltransferase 2, and microsome triglyceride transfer protein activities in a human hepatoma cell line (Wilcox *et al.*, 2001). Hence, the relation between cholesterol metabolism and the components contained in mixUM-GT requires future investigation.

In conclusion, the fermented tea made with third crop green tea leaves and unripe satsuma mandarin fruits, materials not currently effectively utilized, was shown to possess potential hypolipidemic effects. Thus, this new fermented tea may serve as a novel functional food material. Additional studies are needed to elucidate the detailed mechanism underlying the effects of this fermented tea and to identify compounds responsible for improving lipid metabolism.

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