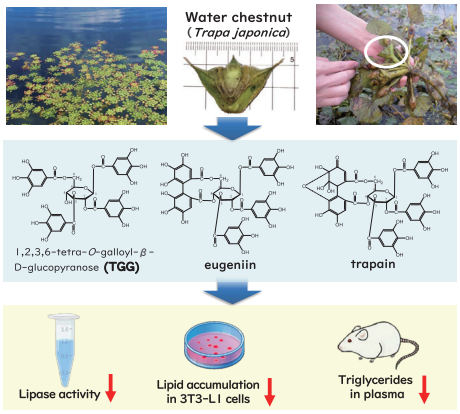
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Hydrolyzed Polyphenols from Water Chestnut (*Trapa japonica*) Suppress Lipase Activity in vitro and Blood Triacylglycerols in Mice

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Abstract: Water chestnut (*Trapa japonica*) is a thorny aquatic floating plant that contains hydrolyzable polyphenols (WCPs) such as eugeniin, 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (TGG), and trapain. Previous studies have revealed that WCPs have antioxidant properties and can suppress postprandial blood glucose elevation in mice and human participants. However, the functionality of WCPs in treating obesity and metabolic syndrome, which have become significant issues in recent years, remains unclear. Here, we aimed to determine the effects of WCPs on lipid metabolism. We initially investigated the effects of WCPs on pancreatic lipase in vitro. Among WCPs, eugeniin and trapain, which contain a hexahydroxybiphenyl group, particularly inhibited lipase activity. We also found that WCPs suppressed fat accumulation in mouse adipose progenitor 3T3-L1 cells. We compared the effects of WCPs on lipid metabolism among groups of mice (n=7 each) fed for four weeks without (standard control and high-fat (HF) diet controls) or with HFWP 0.5 or HFWP 1 supplemented with 0.5 and 1% WCPs, respectively. Subsequently, we examined lipid-related parameters in adipose tissue, liver, and blood. We found significantly lower plasma triacylglycerol content in the HFWP1 than in the HF group. These findings suggest that WCPs may help prevent the onset of obesity and metabolic syndrome.



Key words: water chestnut, polyphenol, lipase, 3T3-L1 cells, triacylglycerols

1 Introduction

Obesity has recently skyrocketed. In 2016, the World Health Organization found that the global population with a body mass index (BMI) ≥ 30 kg/m^2 exceeded 650 million, approximately 13% of the global population aged >18 years[1)]. The Japan Society for the Study of Obesity defines obesity as a BMI ≥ 25 kg/m^2. The ratio of obesity is higher among Japanese males than females, and the rate has essentially doubled between 1973 and 2019[2, 3)]. Obesity is caused by abnormal or excessive fat accumulation that poses a health risk. Japanese individuals with a BMI ≥ 25 kg/m^2 are often affected by at least one obesity-related health disorder, such as glucose intolerance, dyslipidemia,

Abbreviations: **ANOVA**; analysis of variance, **DMEM**; Dulbecco's Modified Eagle's Medium, **EGCG**; (-)-epigallocatechin gallate, **AST**; aspartate aminotransferase, **ALT**; alanine aminotransferase, **HF**; high fat, **HFWP**; addition of water chestnut polyphenols to high-fat diets, **HHBP**; hexahydroxy-biphenyl, **HPLC**; high-performance liquid chromatography, **LDL**; low-density lipoprotein, **TGG**; 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose, **WCP**; water chestnut polyphenol

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and hyperglycemia[4]. Furthermore, the hazard ratio (HR) for death from coronary heart disease and stroke among persons with BMI > 25 kg/m^2 gradually increases[5]. An increase in BMI is associated with higher monthly medical costs[6], leading to the concern that obesity is causing economic losses.

Visceral obesity, in which excess fat accumulates around the intestines in the abdominal cavity, is particularly concerning. In Japan, individuals with a BMI < 25 but an abdominal circumference ≥ 85 cm and ≥ 90 cm for men and women, respectively, along with at least two of the following: blood pressure, blood sugar, and serum lipids exceeding the reference values can be diagnosed with metabolic syndrome. The number of people with metabolic syndrome has increased by approximately 8% since 2008, reaching an estimated 15.16 million people[7]. Lifestyles need to improve in terms of better dietary habits and moderate exercise to prevent and eliminate obesity and metabolic syndrome. However, a sudden change in lifestyle habits through self-care is difficult to achieve and maintain.

As a complementary and alternative approach to diet and exercise therapy, research on food components with functional properties has been active. Among these, many studies have focused on polyphenols. Polyphenols are broadly divided into monomeric and polymeric polyphenols. Among monomeric polyphenols, catechin, a type of flavonoid, exhibits anti-obesity effects in mice[8], rats[9], and humans[10]. Polymeric polyphenols from chestnut husk[11] and persimmon[12] are known to inhibit lipid and bile acid adsorption and are classified as condensed polyphenols. Conversely, less is known about the effects of hydrolyzable polyphenols on lipid metabolism.

Water chestnuts (*Trapa japonica*) float and grow in ponds, swamps, and streams worldwide, bearing a unique thorny fruit in the fall. They are regarded as a nuisance in many parts of Japan, but they are edible and consumed in some areas. The starchy interior of the water chestnuts has a taste similar to chestnuts, hence the name. The husks of water chestnuts are discarded, and little is known about their contents and potential functions. Therefore, we isolated and purified hydrolyzable polyphenols (WCPs) from water chestnut husks and identified them as eugeniin, 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (TGG), and trapain with several gallic acids (Fig. 1)[13]. Trapain, named after the scientific name of water chestnut, is unique to water chestnuts[14]. We previously found that WCPs have antioxidant activities and can suppress postprandial blood glucose elevation in mice and human participants[13, 15]. WCPs also exhibited inhibitory activity against hyaluronidase, elastase, and collagenase, suggesting anti-aging effects[16]. However, the functionality of WCPs on lipid metabolism syndrome remains unclear. Therefore, we aimed to determine the effects of WCPs on lipase and lipid accumulation in 3T3-L1 progenitor adipocytes in vitro and on lipid metabolism in mice fed with a high-fat diet in vivo.

2 Materials and Methods

2.1 Materials

The starchy fruits of wild water chestnuts collected from a stream in Kanzaki City (Saga, Japan) were dried and crushed. Thereafter, the crushed material (100 g) was soaked for 24 h in 2 L of 80% ethanol in Erlenmeyer flasks (2 L) and subsequently passed through No. 1 filter paper (Advantec, Co., Ltd., Tokyo, Japan). The ethanol was evaporated, and the material was filtered again and concentrated using an evaporator. The concentrate was eluted through a DIAION HP20 column with a diameter of 80 × 700 mm (Mitsubishi Chemical Co., Ltd., Tokyo, Japan) with distilled water to remove impurities, followed by 80% ethanol. The eluate was concentrated and dried in an evaporator to obtain crude WCPs. Eugenin, TGG, and trapain were separated and isolated from this extract using high-performance liquid chromatography (HPLC) as described[13].

The crude WCPs were dissolved in ethanol and diluted with buffer or medium as described below to an ethanol

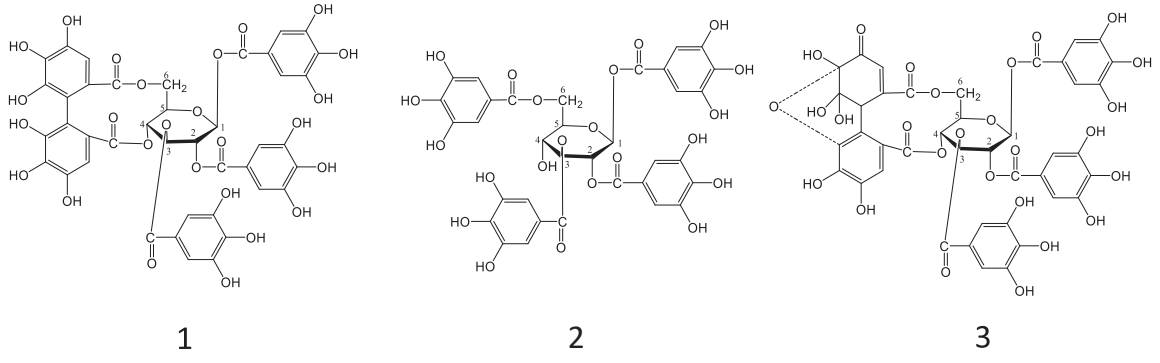


Fig. 1 Structural formula of water chestnut polyphenols. 1, 2, and 3 represent eugeniin, 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose (TGG), and trapain, respectively. WCPs, water chestnut polyphenols.

concentration of 1% or less, subsequently used in experiments on pancreatic lipase activity and lipid accumulation in 3T3 cells. The apparent molar concentration of the crude WCPs is shown as the total molar concentration derived from the content of the purified components and their respective molecular weights.

2.2 Reagents

All special grade and other reagents (unless otherwise stated) and HPLC-grade solvents were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan).

2.3 Lipase activity assay

Porcine pancreatic lipase type II (Merck KGaA, Darmstadt, Germany) (0.05 mg/mL) was dissolved in 0.1 M Tris-HCl buffer (pH 7.5), and the lipase activity was assayed using a Lipase Kit S (Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan). We assayed crude polyphenol, the three purified WCPs, and >98% pure polyphenol (-)-epigallocatechin gallate (EGCG) derived from green tea (Nagara Chemical Industry, Gifu, Japan). All assays were performed in triplicate. The rate of lipase inhibition was calculated as:

$$\text{Lipase inhibition rate}(\%) = 100 - \{(S - SB)/(C - CB)\} \times 100 \quad (1),$$

where S and C represent the absorbance of the sample and water, respectively; SB and CB represent the absorbance of the sample and water blank, respectively.

2.4 Lipid accumulation in 3T3-L1 cells

The lipid accumulation in 3T3-L1 cells was investigated as described previously[17−19]. Briefly, mouse adipose progenitor 3T3-L1 cells (Human Science Foundation, Tokyo, Japan) were seeded in 24-well plates at a constant density of 1×10^5/well and incubated for 3 days at 37℃ under a 5% CO_2 atmosphere. The cells were induced to differentiate into adipocytes using 3-isobutyl-1-methylxanthine, dexamethasone, and insulin provided in Adipogenesis Assay Kits (Cayman Chemical Company, Ann Arbor, MI). Crude WCPs (final concentrations of 12.5, 25, and 50 μg/mL) or EGCG (final concentration of 25 μg/mL) was added to Dulbecco's modified Eagle's medium (DMEM Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 1% penicillin-streptomycin and either 10% fetal calf serum during cell passage or 10% fetal bovine serum to induce differentiation. After incubation for 3 days, the medium was supplemented with fresh samples. At this time, the samples included medium only (untreated control), and progenitor cells (undifferentiated) without differentiation induction. This was incubated for an additional 4 days. Thereafter, the medium supernatant was removed, and the cells were fixed and stained with Oil Red O as described by the manufacturer of the Adipogenesis Assay Kit. Absorbance was measured at 520 nm using a SpectraMax M2e microplate reader (Molecular Devices, LLC., San Jose, CA). All experiments were conducted in triplicate. The lipid accumulation rate was calculated as follows:

$$\text{Lipid accumulation rate}(\%) = (S - BL)/(C - BL) \times 100 \quad (2),$$

where S, BL, and C are the absorbance of the sample solution, undifferentiated progenitor cells, and the untreated control, respectively.

2.5 Assays of lipids in mouse carotid artery, and adipose tissue, liver, and blood

Six-week-old male ICR mice (Japan SLC Inc., Shizuoka, Japan) were fed an MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan) for 7 days and divided into four groups (n = 7 per group) with a similar average body weight. The groups were as follows: control group, high-fat (HF) group, high-fat diet with 0.5% crude WCPs (HFWP0.5) group, and high-fat diet with 1% crude WCPs (HFWP1) group. The control group was fed an AIN-76 powdered diet (CLEA Japan, Inc., Tokyo, Japan). The other three groups were fed a high-fat (HF) AIN-76 diet designed to provide 40% of total energy from lipids, based on previous research[20]. Specifically, 20% of the sucrose in the AIN-76 diet was replaced by a mixture of lard and corn oil. In addition, referring to existing studies with EGCG[21−23], the HFWP0.5 and HFWP1 groups were fed diets in which the sucrose in the HF diet was replaced by 0.5% and 1% (w/w) of crude WCPs, respectively. The detailed composition of each diet is given in Table 1. The controls were fed ad libitum, whereas the HF, HFWP0.5, and HFWP1 groups were pair-fed. After 28 days, mice were euthanized by cervical dislocation without fasting and blood was taken from the carotid artery. Adipose tissue and liver were then removed. The collected blood was supplemented with sodium heparin solution (Ajinomoto Co., Inc., Tokyo, Japan) and then centrifuged at $850 \times g$ for 20 minutes to obtain the supernatant plasma. Lipids in the liver were extracted as described[24], and total lipid content was determined. Triacylglycerols in the liver and blood plasma, as well as total cholesterol and free fatty acids in plasma, were quantified using Triglyceride E-Test-Wako, Cholesterol E-Test-Wako, and NEFA C-Test-Wako, respectively (all from FUJIFILM Wako Pure Chemical Corp.). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were quantified using corresponding kits (FUJIFILM Wako Pure Chemical Corp.). Mouse plasma leptin was evaluated using Mouse/Rat Leptin ELISA kits (Morinaga Institute of Biochemistry Co., Ltd., Kanagawa, Japan), and adiponectin was measured using Levis Polymer Adiponectin-Mouse/Rat ELISA kits (Shibayagi Co., Ltd., Gunma, Japan).

The Nagasaki University Animal Experiment Committee approved the study protocol (Approval No: 1408271169).

Table 1 Composition of experimental diets.

Ingredient	Content (g/100 g)			
	Control	HF	HFWP0.5	HFWP1
Casein	20.0	20.0	20.0	20.0
Corn oil	5.0	20.0	20.0	20.0
Sucrose	50.0	35.0	34.5	34.0
β-Corn starch	15.0	15.0	15.0	15.0
Cellulose	5.0	5.0	5.0	5.0
Mineral mixture[a]	3.5	3.5	3.5	3.5
Vitamin mixture[a]	1.0	1.0	1.0	1.0
DL-Methionine	0.3	0.3	0.3	0.3
Choline tartrate	0.2	0.2	0.2	0.2
Crude WCPs	0	0	0.5	1.0
Total energy				
Energy (kcal/100 g)	374.8	448.5	446.5	444.5
Protein (%)	19.1	16.0	16.0	16.1
Carbohydrate (%)	69.1	44.6	44.3	44.1
Fat (%)	11.8	39.5	39.6	39.8

[a]Mixed with commercial mouse food (AIN-76, CLEA Japan, Inc.). WCPs represent water chestnut polyphenols. HF indicates a high-fat diet; HFWP0.5 and HFWP1 indicate high-fat diets containing 0.5 and 1% crude water chestnut polyphenols, respectively.

The mice were handled during the study according to guidelines outlined in the Animal Welfare Law of Japan, Standards for Breeding, Storage and Pain Relief (Ministry of the Environment of Japan), Basic Guidelines for Animal Experimentation (Ministry of Education, Culture, Sports, Science and Technology of Japan), and Animal Experiment Regulations of Nagasaki University.

2.6 Statistical analysis

The results are expressed as means ± standard deviation (SD). The statistical analysis for significant differences between groups was conducted as follows. First, homogeneity of variance was assessed using Levene's test. When equal variances were confirmed, one-way analysis of variance (ANOVA) was performed. If significant differences were detected, Tukey–Kramer's post hoc test was employed for multiple comparisons. In cases where the assumption of homogeneity of variance was violated, data were log-transformed and Levene's test was repeated, followed by the aforementioned procedure. For all statistical analyses, $p<0.05$ was considered statistically significant, while $p<0.01$ was deemed highly significant. Statistical analyses were performed using Excel Statistics (Ver. 4.07, Social Information Service Co., Ltd., Tokyo, Japan).

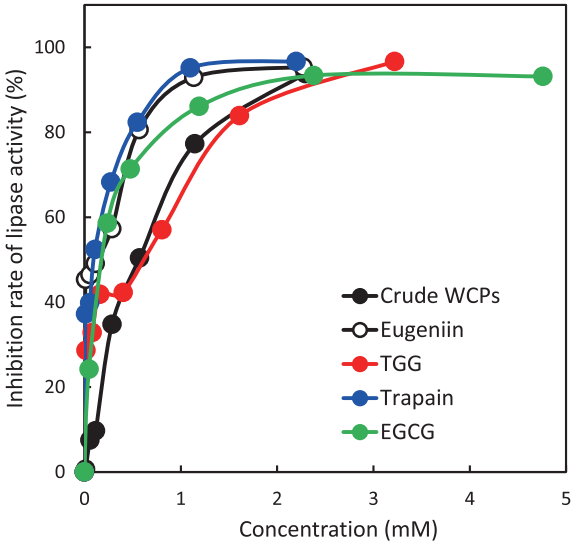


Fig. 2 Effect of concentrations of WCPs on the inhibitory activity of lipase. TGG and EGCG indicate 1,2,3,6-tetra-*O*-galloyl-β-D-glucopyranose and (-)-epigallocatechin gallate, respectively. WCPs: water chestnut polyphenols.

3 Results

3.1 Effects of WCPs on lipase

The percentage inhibition of lipase activity of the three WCPs, crude WCPs, and EGCG was determined at different

concentrations, and is shown in Fig. 2. These samples dependently inhibited lipase activity with increasing concentration, with the inhibition effect constant at a specific concentration. The concentration of 50% inhibition of lipase activity (IC_{50}) was calculated from the inhibition curve of each sample using the best-fit regression equation. The experiment was conducted in triplicate for each sample and the mean and standard deviation of the IC_{50} values obtained are listed in Table 2.

Table 2 Inhibitory effect of WCPs on lipase.

Sample	IC_{50}	Unit
Purified WCPs		
TGG	414 ± 71**	μM
Eugeniin	163 ± 57	μM
Trapain	141 ± 7	μM
Crude WCPs	816 ± 41	μg/mL
	529 ± 27[a]**	μM
EGCG	188 ± 8	μM

The IC_{50} value refers to the concentration at which 50% of lipase activity is inhibited. Data presented are mean ± standard deviation (SD, n=3). WCPs represent water chestnut polyphenols. The "a" represents the molar concentration derived from the content of the purified components and their respective molecular weights. The statistical analysis used was Dunnett's method, testing for significant differences versus (-)-epigallocatechin gallate (EGCG) (**$p<0.01$).

Eugeniin and trapain inhibited lipase activity more effectively than TGG. The IC_{50} of crude polyphenol was 816 ± 41 μg/mL. The contents of TGG, eugeniin, and trapain in crude polyphenols were 15.1 ± 0.5, 19.6 ± 0.4, and 23.7 ± 1.3 g/100 g, respectively, indicating a summed apparent molar concentration of 529 ± 27 μM. The IC_{50} of the crude polyphenol was slightly higher than that of TGG. The antioxidant properties and significant inhibitory effects on lipase activity of EGCG have been established. The IC_{50} was significantly higher for the TGG and the crude polyphenol and lower for eugeniin and trapain compared to EGCG. This finding implies that eugeniin and trapain are more powerful inhibitors of lipase activity than EGCG.

3.2 Effect of WCPs on lipid accumulation in 3T3-L1 cells

Figure 3 shows 3T3-L1 cells (red area) stained with Oil Red O to detect adipocytes. Although we did not directly examine cytotoxicity to 3T3-L1 cells in this study, we believe that it is unlikely that the WCPs caused significant toxicity to the cells, as the addition of the samples did not alter cell morphology or cause gaps in the culture plates. Cells treated with crude WCPs (Figs. 3C and 3D) appeared less red area than the control (Fig. 3B), indicating that crude WCPs reduced the amount of adipocytes present. Figure 4 shows the lipid accumulation rate of 3T3-L1 cells calculated from equation (2); Cells treated with WCPs showed significantly lower fat accumulation rates than the control at all concentrations tested ($p<0.01$). Notably, the inhibitory effect of the crude WCPs at 12.5 μg/mL was comparable to that of EGCG at 25 μg/mL, with no statisti-

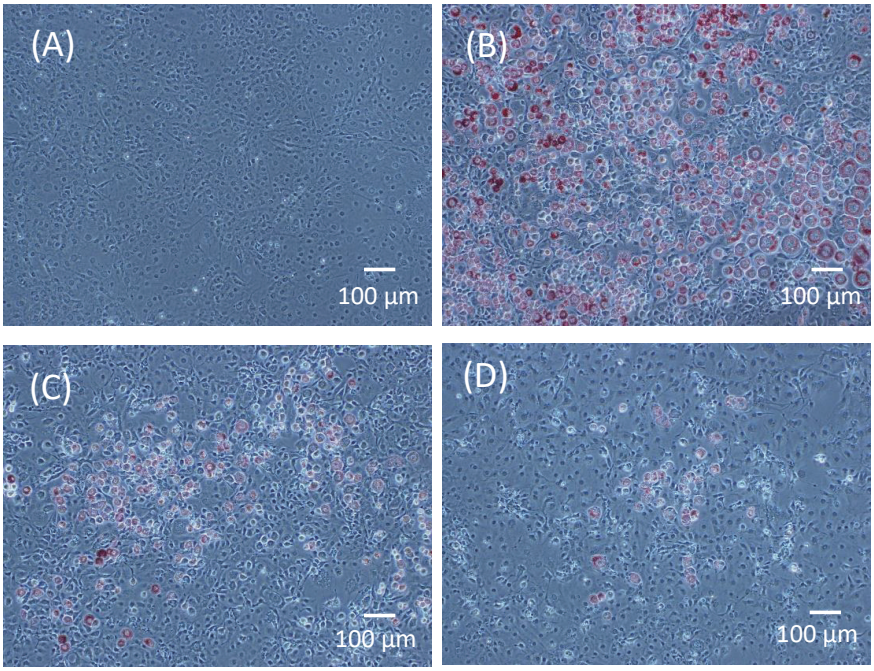


Fig. 3 Detection of lipids in 3T3-L1 cells stained with Oil Red O. (A) Undifferentiated progenitor cells. (B) Control cells. Cells incubated with (C) 25 and (D) 50 μg/mL of crude WCPs (water chestnut polyphenols).

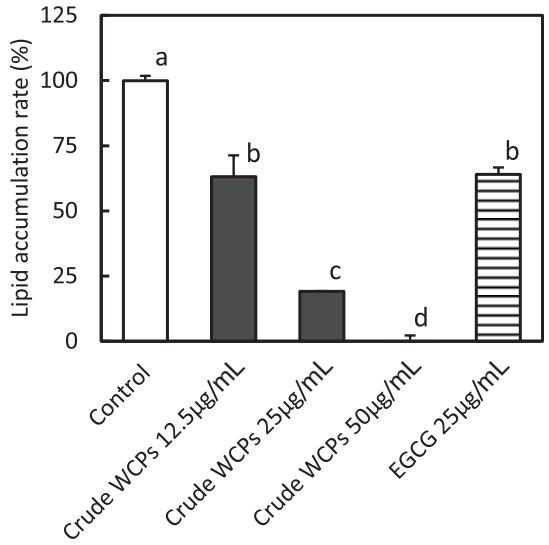


Fig. 4 Inhibitory effect of crude WCPs on lipid accumulation in 3T3-L1 cells. WCPs and EGCG indicate water chestnut polyphenols and (-)-epigallocatechin gallate, respectively. Error bars represent standard deviation (n = 3). Significant differences ($p < 0.01$) are indicated between the different signs.

cally significant difference. The total (apparent) concentration of the three polyphenols in the WCPs was 8.1 μM. This is lower than the effective concentration of EGCG alone (54.5 μM), indicating that WCPs effectively inhibit fat accumulation at lower concentrations.

3.3 Effects of WCPs on lipid metabolism in mice

Table 3 shows the food intake, body weight gain, tissue weight, and blood biochemical profiles of mice treated with crude WCPs. The total food intake of the control group was significantly higher than that of the other groups, but there was no significant difference in total energy intake among all groups. Body weight gain and liver weight did not significantly differ throughout the study. However, the amount of epididymal and perirenal fat was significantly more abundant in the HFWP0.5 than in the control but did not significantly differ from the HF group. Total lipid weight or triacylglycerol and cholesterol contents in the liver, as well as plasma AST and ALT levels that indicate liver function, did not significantly differ among the groups. The total plasma cholesterol content was significantly lower in the HFWP1 group than in the HFWP0.5 group, while total cholesterol did not significantly differ between the control and HF groups. The HFWP0.5 group had significantly lower free fatty acid levels than the controls but did not significantly differ from the HF groups. Levels of adiponectin and leptin, which are involved in fatty acid production, also did not significantly differ. Conversely, plasma triacylglycerol levels were significantly lower in the HFWP1 group than in the control and HF groups (Fig. 5).

4 Discussion

We initially determined that crude and purified WCPs (TGG, eugeniin, and trapain) inhibited pancreatic lipase activity in vitro more effectively than EGCG. The inhibitory effect of EGCG might be influenced by the pyrogallol and galloyl groups in its B-ring[25, 26]. Interaction between EGCG and lipase at a ratio of 1:1 alters the molecular structure of lipase and decreases enzyme activity[27]. Polymerized polyphenols in Oolong tea inhibit lipase activity more effectively than EGCG, suggesting that intramolecular galloyl groups are important[28]. Galloyl groups are more abundant in WCPs than in EGCG; thus, these groups might be responsible for the more potent inhibition of lipase activity. The IC_{50} was lower for eugeniin and trapain compared to EGCG, although the difference did not reach significance. The IC_{50} for TGG was higher than that for eugeniin and trapain. This may be explained by their hexahydroxy-biphenyl (HHBP) groups, comprising coupled gallic acids that TGG lacks. Eugeniin and trapain may be more likely to interact with lipase because of increased planarity caused by HHBPs. The WCPs herein inhibited pancreatic lipase activity, which might lead to inhibited lipid digestion and absorption from the small intestine. A slower rate of lipid absorption is thought to reduce the rapid postprandial rise in blood triacylglycerols.

We found that crude WCPs suppressed lipid accumulation in 3T3-L1 cells and exerted inhibitory effects at about one-seventh of the molar concentration required by EGCG. Catechins in green tea are thought to prevent lipid accumulation by inhibiting the expression of peroxisome proliferator-activated receptor γ, which activates peroxisome proliferator and cytosine-cytosine-adenosine-adenosine-thymidine box motif/enhancer binding protein, which are transcription factors involved in the differentiation of pre-3T3-L1 adipocytes into adipocytes[29, 30]. Catechins might also inhibit adipocyte differentiation via the insulin signaling pathway by decreasing the activity of forkhead transcription factor class O1 and sterol regulatory element binding protein[31]. Here, WCPs inhibited fat accumulation more effectively than catechins, suggesting reduced expression of adipocyte differentiation transcription factors and direct inhibition of adipocyte differentiation via the insulin signaling pathway.

Based on these findings in vitro, we investigated the effects of WCPs in vivo using mice. None of the liver components significantly differed between the HFWP, and the control and HF groups, indicating that WCPs did not affect the liver. Only triacylglycerols among the plasma components were significantly lower in the HFWP1 group than in the control and HF groups (Fig. 5). Monomeric polyphe-

Table 3 Effect of water chestnut polyphenol administration on food intake, body weight, and biochemical data in mice.

Item	Group			
	Control	HF	HFWP0.5	HFWP1
Total food intake (g)	175 ± 10^{a}	138 ± 11^{b}	133 ± 8^{b}	139 ± 16^{b}
Total energy intake (kcal)	643 ± 38	610 ± 50	583 ± 36	609 ± 71
Body weight gain (g)	15.4 ± 2.2	16.7 ± 2.4	17.1 ± 2.7	15.3 ± 2.2
Organ weight (g)				
Liver	2.28 ± 0.35	2.14 ± 0.19	2.07 ± 0.23	2.03 ± 0.20
Epididymal fat	1.39 ± 0.39^{a}	2.01 ± 0.61^{ab}	2.43 ± 0.47^{b}	1.75 ± 0.66^{ab}
Perinephric fat	0.62 ± 0.19^{A}	0.83 ± 0.26^{AB}	0.95 ± 0.11^{B}	0.81 ± 0.23^{AB}
Components in the liver (mg/g liver)				
Total lipid	61.5 ± 34.6	78.8 ± 23.4	55.6 ± 22.4	73.5 ± 30.1
Triacylglycerols	45.6 ± 28.7	61.0 ± 29.4	43.4 ± 18.9	54.3 ± 20.2
Total cholesterol	3.19 ± 1.78	4.06 ± 1.23	2.75 ± 0.76	4.50 ± 3.37
Components in plasma				
AST (IU/L)	352 ± 123	241 ± 60	320 ± 109	438 ± 304
ALT (IU/L)	21.7 ± 7.1	25.7 ± 13.9	23.3 ± 4.8	77.1 ± 110.5
Total cholesterol (mg/dL)	145 ± 27^{AB}	158 ± 33^{AB}	174 ± 21^{A}	113 ± 47^{B}
NEFA (mEq/L)	0.93 ± 0.09^{A}	0.78 ± 0.19^{AB}	0.69 ± 0.12^{B}	0.72 ± 0.21^{AB}
Adiponectin (μg/mL)	10.5 ± 5.5	7.4 ± 3.1	7.6 ± 2.3	8.8 ± 3.2
Leptin (ng/mL)	12.0 ± 10.4	15.2 ± 8.4	14.1 ± 7.9	10.9 ± 7.7

HF indicates a high-fat diet; HFWP0.5 and HFWP1 indicate high-fat diets containing 0.5 and 1% crude water chestnut polyphenols, respectively. NEFA, AST, and ALT indicate non-esterified fatty acid (free fatty acid), aspartate aminotransferase, and alanine aminotransferase, respectively. All data are expressed as mean ± standard deviation. Significant differences (uppercase: $p < 0.05$, lowercase: $p < 0.01$) are indicated between the different signs.

nols, such as EGCG[23, 32], condensed polymerized polyphenols in oolong tea[33], and polyphenols in black tea[34] have been reported to reduce blood triacylglycerol levels. It has been reported that mice fed a high-fat diet supplemented with 1% EGCG for four weeks showed a significant reduction in blood TG compared to the high-fat diet in the control group[23]. However, because of the different experimental conditions, these results cannot be directly compared to the results of the present study. On the other hand, catechins with galloyl groups have been shown to lower blood TG[35], and similar blood TG lowering effects were observed in WCPs, suggesting that it is important that WCPs contain three or more galloyl or HHBP groups.

Although we did not directly measure TG levels in feces in this study, an increase in fecal lipids and a decrease in blood TG have been reported in a previous study using water chestnut extract[36]. In this paper, ellagic acid is inferred to be an involved component. Ellagic acid is a polyphenol produced by hydrolysis of ellagitannins, such as

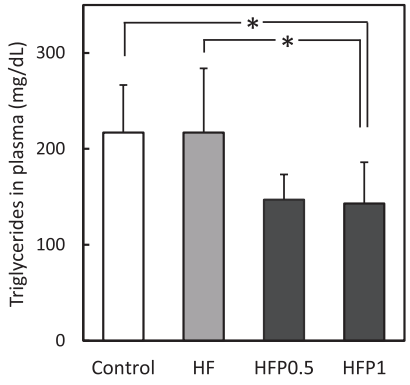


Fig. 5 Inhibitory effect of crude WCPs administration on plasma triacylglycerol levels in mice. HF indicates a high-fat diet; HFWP0.5 and HFWP1 indicate high-fat diets containing 0.5 and 1% crude WCPs, respectively. WCPs: water chestnut polyphenols. Error bars represent standard deviation (n = 7). * $p < 0.05$.

those found in WCPs. It has been reported that pomegranate extract containing ellagic acid inhibits lipase activity[37], and WCPs also contain a functional group similar to ellagic acid.

Considering the lipase inhibitory effect of WCPs, the in vivo blood TG reduction effect observed in this study, and the reports of increased fecal lipid excretion in previous studies, the following mechanism can be inferred: Lipase inhibition by WCPs inhibits hydrolysis of dietary TG, resulting in decreased TG absorption from the intestinal tract and thus suppresses the increase in blood TG.

Furthermore, the results of in vitro experiments using 3T3-L1 cells and in vivo experiments using ICR mice were not in agreement. This may be due to factors such as the effective concentration of WCPs and the effects of metabolism and absorption in vivo. Specifically, it has been reported that our experiments using 3T3-L1 cells showed a fat accumulation effect, but the addition of 4 μM of eugeniin did not suppress lipid accumulation[38]. In addition, although we did not measure plasma WCPs concentrations in this study, it is possible that the amount of WCPs ingested was insufficient to exert the adipose tissue-reducing effect in vivo. In the future, it will be important to evaluate the absorption rate of WCPs in the body and to calculate the amount of WCP intake required to exert lipid-improving effects in vivo. Although the mechanism of inhibition of fat accumulation in 3T3-L1 cells has not been fully elucidated, a 20% ethanol extract of water chestnut activates cAMP, promotes degradation of triacylglycerol in adipocytes, and decreases SREBP1c and FAS[39]. It has been reported that it suppresses fat synthesis and intracellular lipid accumulation by decreasing protein expression. Therefore, the mechanism of suppression of lipid accumulation in 3T3-L1 cells in the present study may also be due to the promotion of lipolysis and suppression of fat synthesis.

Polyphenols pass through the digestive tract, where they are enzymatically hydrolyzed, absorbed into the intestine, and then conjugated to glucuronide and sulfate by phase II enzymes in epithelial cells and the liver; they are not readily taken up by vascular cells[40]. Polyphenols that are not absorbed enter the large intestine, where indigenous bacteria cause reactions such as ring cleavage, methylation of hydroxyl groups, dehydroxylation, and hydrolysis. These reactions produce degradation products, such as phenolic acids, valerolactone, and fermentation products, such as short-chain fatty acids[40–42]. These products are absorbed by the large intestine, where they express physiological activity in vivo[40–42]. The degradation or fermentation products of WCPs, which are absorbed by the large intestine, may express physiological activity.

Total cholesterol in plasma was significantly higher in the HFWP0.5 group than in the HFWP1 group, while total cholesterol in the liver was approximately 40% lower in the HFWP0.5 group. These results suggest that cholesterol release from the liver may have been enhanced in the HFWP0.5 group; cholesterol metabolic pathways may be differentially affected by concentrations of WCPs, with higher concentrations inhibiting cholesterol release from the liver. Recent studies have reported that eugeniin reduces intracellular cholesterol levels and increases LDL receptor expression in a hepatocyte model, while suppressing the expression of enzymes involved in cholesterol synthesis[43]. Eugeniin may improve overall cholesterol metabolism by modulating intracellular cholesterol levels through increasing cholesterol uptake, inhibiting synthesis, and promoting efflux. In the present study, it is possible that WCPs promoted cholesterol uptake into the liver and reduced blood cholesterol levels.

The study has some limitations. First, the cytotoxicity of WCPs to 3T3-L1 cells was not evaluated in detail. Future studies should quantitatively evaluate cell viability and cell proliferation rates. These additional experiments will confirm whether the observed effects are due to cytotoxicity, or are a true biological response. Direct measurement of fecal TG levels and changes in intestinal microbiota should be investigated to elucidate the mechanism of inhibition of fat accumulation by WCPs. In addition, further molecular biological approaches such as expression analysis of fat synthesis-related genes, measurement of lipolysis-related enzyme activity, and analysis of insulin signaling pathways are needed. Furthermore, the plasma concentration of WCPs was not measured, and the intake of WCPs may have been insufficient to exert adipose tissue reduction effects. Future studies are needed to evaluate the absorption of WCPs and in different animal models. Finally, the mechanism of cholesterol at the molecular level needs to be elucidated and the concentration-dependent effects of WCPs need to be clearly demonstrated.

5 Conclusion

This study investigated the effects of water chestnut-derived polyphenols (WCPs) on lipid metabolism and shows interesting results: in vitro experiments, WCPs effectively inhibited lipase activity, with eugeniin and trapain having particularly potent effects; in experiments using 3T3-L1 cells, WCPs inhibited fat accumulation at lower concentrations than EGCG. In in vivo experiments using mice, blood triacylglycerol levels were significantly reduced in the group in which 1% of WCPs was added to a high-fat diet. This suggests that the mechanism of action of WCPs is inhibition of TG absorption from the intestinal tract through lipase inhibition. An effect on cholesterol metabolism was also observed, but the effect was concentration-dependent and requires further study. In general, WCPs show promising effects in improving lipid metabolism, but further

studies are essential to elucidate the details of the mechanism of action and to identify the optimal intake.

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Author Contributions

M. Y. conceptualization, methodology, validation, formal analysis, investigation, data curation, writing - original draft, visualization, supervision, and project administration. K. Y. methodology, validation, and writing - review & editing. D. O. methodology, formal analysis, investigation, data curation, writing - review & editing, and visualization. M. S. and A. M.-T. data curation, and writing - review & editing. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

1) Obesity and overweight (2021) [Internet]. Genève, Switzerland: World Health Organization. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. Accessed 19 January 2024.
2) Japan Society for the Study of Obesity. *Guidelines for the Diagnosis and Treatment of Obesity 2022*, Life Science Publishing Co., 2022, p. 29 (2022).
3) National Health and Nutrition Examination Survey. Medical survey. Health Japan 21 (2nd) Analysis and Evaluation Project [Internet]. Osaka, Japan: National Institute of Health and Nutrition. https://www.nibiohn.go.jp/eiken/kenkounippon21/eiyouchousa/keinen_henka_shintai.html. Accessed 19 January 2024.
4) Examination Committee of Criteria for 'Obesity Disease' in Japan; Japan Society for the Study of Obesity. New Criteria for 'Obesity Disease' in Japan. *Circ. J.* **66**, 987-992 (2002). doi: 10.1253/circj.66.987
5) The Global BMI Mortality Collaboration. Body-mass index and all-cause mortality: Individual-participant-data meta-analysis of 239 prospective studies in four continents. *Lancet* **388**, 776-786 (2016). doi: 10.1016/S0140-6736(16)30175-1
6) Fujita, M.; Sato, Y.; Nagashima, K.; Takahashi, S.; Hata, A. Medical costs attributable to overweight and obesity in Japanese individuals. *Obes. Res. Clin. Pract.* **12**, 479-484 (2018). doi: 10.1016/j.orcp.2018.06.002
7) National Health and Nutrition Examination Survey. Annual changes in current values. Health Japan 21 (2nd) Analysis and Evaluation Project [Internet]. Osaka, Japan: National Institute of Health and Nutrition. https://www.nibiohn.go.jp/eiken/kenkounippon21/kenkounippon21/genjouchi.html. Accessed 19 January 2024.
8) Bose, M.; Lambert, J.D.; Ju, J.; Reuhl, K.R.; Shapses, S.A. et al. The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. *J. Nutr.* **138**, 1677-1683 (2008). doi: 10.1093/jn/138.9.1677
9) Snoussi, C.; Ducroc, R.; Hamdaoui, M.H.; Dhaouadi, K.; Abaidi, H. et al. A. Green tea decoction improves glucose tolerance and reduces weight gain of rats fed normal and high-fat diet. *J. Nutr. Biochem.* **25**, 557-564 (2014). doi: 10.1016/j.jnutbio.2014.01.006
10) Rains, T.M.; Agarwal, S.; Maki, K.C. Anti-obesity effects of green tea catechins: A mechanistic review. *J. Nutr. Biochem.* **22**, 1-7 (2011). doi: 10.1016/j.jnutbio.2010.06.006
11) Kawasaki, K.; Shiromizu, T.; Katsui, M. Suppressive effect of polyphenols extracted from chestnut skins on lipid Absorption in rats. *Nippon Shokuhin Kagaku Kogaku Kaishi* **56**, 545-548 (2009). doi: 10.3136/nskkk.56.545
12) Hamauzu, Y.; Suwannachot, J. Non-extractable polyphenols and in vitro bile acid-binding capacity of dried persimmon (*Diospyros kaki*) fruit. *Food Chem.* **293**, 127-133 (2019). doi: 10.1016/j.foodchem.2019.04.092
13) Yasuda, M.; Yasutake, K.; Hino, M.; Ohwatari, H.; Ohmagari, N. et al. Inhibitory effects of polyphenols from water chestnut (*Trapa japonica*) husk on glycolytic enzymes and postprandial blood glucose elevation in mice. *Food Chem.* **165**, 42-49 (2014). doi: 10.1016/j.foodchem.2014.05.083
14) Nonaka, G.; Matsumoto, Y.; Nishioka, I. Trapain, a new hydrolyzable tannin from *Trapa japonica* FLEROV. *Chem. Pharm. Bull.* **29**, 1184-1187 (1981). doi: 10.1248/cpb.29.1184
15) Yasuda, M.; Yasutake, K.; Ohmagari, N.; Saiki, M.; Kojima, Y. Properties of polyphenols in hot water extract of water chestnut husk and suppressive effect on postprandial blood glucose elevation in humans. *Food Preserv. Sci.* **44**, 179-187 (2018). doi: 10.5891/jafps.44.179
16) Yasuda, M.; Ikeoka, M.; Kondo, S. Skin-related enzyme inhibitory activity by hydrolyzable polyphenols in water chestnut (*Trapa natans*) husk. *Biosci. Biotech.*

Biochem. **85**, 666-674(2021). doi: 10.1093/bbb/zbaa076
17) Shoji, T.; Kobori, M.; Shinmoto, H.; Yanagida, A.; Kanda, T. et al. Inhibitory effects of apple polyphenols on differentiation of 3T3-L1 cells into adipocytes. *Food Sci. Technol. Res.* **6**, 119-121(2000). doi: 10.3136/fstr.6.119
18) Iwashita, K.; Yamaki, K.; Tsushida, T. Effect of flavonoids on the differentiation of 3T3-l1 adipocytes. *Food Sci. Technol. Res.* **7**, 154-160(2001). doi: 10.3136/fstr.7.154
19) Iwashita, K.; Yamaki, K.; Tsushida, T. Mioga(*Zingiber mioga Rosc.*)extract prevents 3T3-L1 differentiation into adipocytes and obesity in mice. *Food Sci. Technol. Res.* **7**, 164-170(2001). doi: 10.3136/fstr.7.164
20) Hariri, N.; Thibault, L. High-fat diet-induced obesity in animal models. *Nutr. Res. Rev.* **23**, 270-299(2010). doi: 10.1017/S0954422410000168
21) Klaus, S.; Pültz, S.; Thöne-Reineke, C.; Wolfram, S. Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. *Int. J. Obes.* **29**, 615-623(2005). doi: 10.1038/sj.ijo.0802926
22) Friedrich, M.; Petzke, K.J.; Raederstorff, D.; Wolfram, S.; Klaus, S. Acute effects of epigallocatechin gallate from green tea on oxidation and tissue incorporation of dietary lipids in mice fed a high-fat diet. *Int. J. Obes.* **36**, 735-743(2012). doi: 10.1038/ijo.2011.136
23) Zhou, J.; Mao, L.; Xu, P.; Wang, Y. Effects of(-)-epigallocatechin gallate(EGCG)on energy expenditure and microglia-mediated hypothalamic inflammation in mice fed a high-fat diet. *Nutrients* **10**, 1681(2012). doi: 10.3390/nu10111681
24) Takagi, S.; Oikawa, D.; Ikeda, H.; Tateiwa, N.; Koba, K. et al. The relationship between liver lipid accumulation and changes in plasma amino acids in mice challenged with carbon tetrachloride. *J. Anim. Res. Nutr.* **1**, 1-6(2016). doi: 10.21767/2572-5459.100012
25) Rahim, A.T.M.A.; Takahashi, Y.; Yamaki, K. Mode of pancreatic lipase inhibition activity in vitro by some flavonoids and non-flavonoid polyphenols. *Food. Res. Int.* **75**, 289-294(2015). doi: 10.1016/j.foodres.2015.05.017
26) Wang, S.; Sun, Z.; Dong, S.; Liu, Y.; Liu, Y. Molecular interactions between(-)-epigallocatechin gallate analogs and pancreatic lipase. *PLOS ONE* **9**, e111143(2014). doi: 10.1371/journal.pone.0111143
27) Wu, X.; He, W.; Yao, L.; Zhang, H.; Liu, Z. et al. J. Characterization of binding interactions of(-)-epigallocatechin-3-gallate from green tea and lipase. *J. Agric. Food Chem.* **61**, 8829-8835(2013). doi: 10.1021/jf401779z
28) Nakai, M.; Fukui, Y.; Asami, S.; Toyoda-Ono, Y.; Iwashita, T. et al. Inhibitory effects of oolong tea polyphenols on pancreatic lipase in vitro. *J. Agric. Food Chem.* **53**, 4593-4598(2005). doi: 10.1021/jf047814+
29) Furuyashiki, T.; Nagayasu, H.; Aoki, Y.; Bessho, H.; Hashimoto, T. et al. Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARg2 and C/EBPa in 3T3-L1 cells. *Biosci. Biotechnol. Biochem.* **68**, 2353-2359(2004). doi: 10.1271/bbb.68.2353
30) Chan, C.Y.; Wei, L.; Castro-Muñozledo, F.; Koo, W.L. (-)-Epigallocatechin-3-gallate blocks 3T3-L1 adipose conversion by inhibition of cell proliferation and suppression of adipose phenotype expression. *Life Sci.* **89**, 779-785(2011). doi: 10.1016/j.lfs.2011.09.006
31) Kim, H.; Hiraishi, A.; Tsuchiya, K.; Sakamoto, K. (-)Epigallocatechin gallate suppresses the differentiation of 3T3-L1 preadipocytes through transcription factors FoxO1 and SREBP1c. *Cytotechnology* **62**, 245-255(2010). doi: 10.1007/s10616-010-9285-x
32) Lee, M.S.; Kim, C.T.; Kim, Y. Green tea(-)-epigallocatechin-3-gallate reduces body weight with regulation of multiple genes expression in adipose tissue of diet-induced obese mice. *Ann. Nutr. Metab.* **54**, 151-157(2009). doi: 10.1159/000214834
33) Toyoda-Ono, Y.; Yoshimura, M.; Nakai, M.; Fukui, Y.; Asami, S. et al. Suppression of postprandial hypertriglyceridemia in rats and mice by oolong tea polymerized polyphenols. *Biosci. Biotechnol. Biochem.* **71**, 971-976(2007). doi: 10.1271/bbb.60635
34) Matsumoto, N.; Okushio, K.; Hara, Y. Effect of black tea polyphenols on plasma lipids in cholesterol-fed rats. *J. Nutr. Sci. Vitaminol.* **44**, 337-342(1998). doi: 10.3177/jnsv.44.337
35) Suzuki, Y.; Unno, T.; Kobayashi, M.; Nozawa, A.; Sagesaka, Y. et al. Dose-dependent suppression of tea catechins with a galloyl moiety on postprandial hypertriglyceridemia in rats. *Biosci. Biotechnol. Biochem.* **69**, 1288-1291(2005). doi: 110.1271/bbb.69.1288
36) Kang, H.-G.; Bashir, K.M.I.; Kim, K.-Y.; Shin, S.; Choi, M.-W. et al. Evaluation of dose-dependent obesity and diabetes-related complications of water chestnut (Fruit of *Trapa japonica*)extracts in type II obese diabetic mice induced by 45% kcal high-fat diet. *Medicina* **58**, 189(2022). doi: 10.3390/medicina58020189
37) Hadrich, F.; Cherif, S.; Gargouri, Y.T.; Adel, S. Antioxidant and lipase inhibitory activities and essential oil composition of pomegranate peel extracts. *J. Oleo Sci.* **63**, 515-525(2014). doi: 10.5650/jos.ess13163
38) Nobushi, Y.; Wada, T.; Koike, Y.; Kaneko, H.; Shimba, S. et al. Inhibitory effects of hydrolysable tannins on lipid accumulation in 3T3-L1 cells. *Biol. Pharm. Bull.* **45**, 1458-1465(2022). doi: 10.1248/bpb.b22-00227
39) Park, S.-J.; Lee, M.; Kim, K.-Y.; Shin, S.; Choi. M.-W. et al. Trapa japonica Flerov extract prevents obesity by regulating adipogenesis and lipolysis in differentiated

3T3-L1 cells. *Appl. Sci.* **12**, 290(2022). doi: 10.3390/app12010290
40) Murota, K.; Nakamura, Y.; Uehara, M. Flavonoid metabolism: The interaction of metabolites and gut microbiota. *Biosci. Biotechnol. Biochem.* **82**, 600-610 (2018). doi: 10.1080/09168451.2018.1444467
41) Chen, H.; Sang, S. Biotransformation of tea polyphenols by gut microbiota. *J. Funct. Foods* **7**, 26-42 (2014). doi: 10.1016/j.jff.2014.01.013
42) Valdés, L.; Cuervo, A.; Salazar, N.; Ruas-Madiedo, P.; Gueimonde, M. et al. The relationship between phenolic compounds from diet and microbiota: impact on human health. *Food Funct.* **6**, 2424-2439(2015). doi: 10.1039/c5fo00322a
43) Ye, Y.; Takeuchi, A.; Kawaguchi, Y.; Matsuba, S.; Zhang, N. et al. Eugeniin improves cholesterol metabolism in HepG2 cells and Caco-2 cells. *Biosci. Biotechnol. Biochem.* **88**, 97-106(2024). doi: 10.1093/bbb/zbad158

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