

ORIGINAL**Treatment with indigo plant (*Polygonum tinctorium* Lour) improves serum lipid profiles in Wistar rats fed a high-fat diet**Hiroko Tsuji^{1,2}, Maki Kondo², Wataru Odani², Tasuku Takino², Risako Takeda^{1,2}, and Tohru Sakai¹¹Department of Public Health and Applied Nutrition, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima 770-8503, Japan, ²Faculty of Human Life Science, Shikoku University, Oujin-cho 123-1, Tokushima 771-1192, Japan

Abstract : We investigated the effects of *Polygonum tinctorium* Lour (PTL), a plant commonly known as indigo, on biological parameters in an animal model of high-fat diet-induced obesity. Wistar rats fed a high-fat diet and treated with PTL showed lower serum levels of triglycerides and total cholesterol levels and a higher serum levels of HDL cholesterol than those in Wistar rats fed a high-fat diet without PTL treatment. The weight of mesenteric fat in PTL-treated rats was decreased compared to that in control rats not treated with PTL. In addition, energy metabolic rate in the dark period, but not in the light period, in PTL-treated rats was higher than that in control rats. Although a significant difference was not observed, body weight in PTL-treated rats tended to be decreased compared to that in control rats. The results show that PTL improves serum lipid profiles in Wistar rats with high-fat diet-induced obesity. *J. Med. Invest.* 67:158-162, February, 2020

Keywords : *Polygonum tinctorium* Lour, serum lipids, high-fat diet, metabolic rate, rats

INTRODUCTION

The incidence of metabolic syndrome is increasing worldwide because of the increasing numbers of people with a sedentary lifestyle and people who overeat. Metabolic syndrome is associated with increased risks of cardiovascular diseases and other diseases such as type II diabetes. (1). Obesity is a crucial factor for the development of metabolic syndrome. In the United States, obesity is responsible for approximately 300,000 deaths each year (2). Individuals who are obese are also susceptible to infections and are more likely to develop serious complications from common infections (3). For these reasons, much attention has been paid to the development of functional food for control of body weight.

Indigo plants, which originated in South Vietnam, have been cultivated for producing dye in various parts of the world. Indigo plants are the special local products in Tokushima Prefecture in Japan. Indigo plants have been used to produce dye for paper and cloth. *Polygonum tinctorium* Lour (PTL) is the scientific name for indigo plants. The most well-known property of PTL is its anti-bacterial effect (4, 5). In addition to its anti-bacterial function, PTL has been shown to have anti-inflammatory effects (6, 7), antioxidant activities (8) and anti-cancer effects (9). Since Indigo plants have been used as natural medicine for a long time in Asia, it is possible that Indigo plants have other unknown physiological properties.

In this study, we investigated the effects of PTL on body composition and biochemical markers in rats because there has been no study in which the effects of PTL were investigated in an animal model of diet-induced obesity.

MATERIALS AND METHODS*Rats and diet*

Male Wistar rats (Charles River Lab., Yokohama, Japan) were maintained in a 12-h light : dark cycle at $24 \pm 2^\circ\text{C}$ and $55 \pm 10\%$ relative humidity. Because we examined the effects of *Polygonum tinctorium* Lour (PTL) on adult stage of rat, 5-weeks-old rats (150-160 g) were purchased and given AIN93 standard diet (Oriental Yeast Co., Ltd., Chiba, Japan) until 12 weeks old. The composition of experimental food for the rats was 25.7% casein, 23.5% α -starch (Oriental Yeast Co.), 9% sucrose (Mitsui Sugar Co., Ltd., Osaka, Japan), 23.8% lard (Oriental Yeast Co.), 7% soy bean oil (WAKO, Osaka, Japan), 5% cellulose, 3.5% mineral mixture, 1% vitamin mixture (Oriental Yeast Co.), 0.3% DL-methionine (WAKO), 0.25% choline bitartrate (WAKO), 0.3% L-cystein (WAKO), 0.0014% *tert*-butylhydroquinone (WAKO) and 1% dextrin (WAKO). PTL was supplied as a powder from BON ARM Co. Ltd. (Tokushima, Japan). PTL contains about 2% of polyphenols and the major components are shown in Table 1. In the experimental diet, PTL was added to the control diet at a dose of 1.0% (W/W) instead of dextrin. All experimental procedures were approved by the Animal Research Committee of the Shikoku University.

Table 1. Polyphenol contents in PTL¹

	Content (per 100 g PTL)
Total polyphenols	2.41 g
Tryptanthrin	30.0 mg
Kaempferol	24.0 mg
Indirubin	21.0 mg
Quercetin	2.5 mg
Caffeic acid	2.0 mg
Tannic acid	2.05 g

¹Analysis of polyphenol contents in PTL were done by the Japan Food Analysis Center Foundation as described in the MATERIALS AND METHODS.

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Determination of the polyphenol content and type in PTL was outsourced to the Japan Food Analysis Center Foundation (Tokyo, Japan). Analytical method for tryptanthrin and indirubin was liquid chromatography tandem mass spectrometry analyses, for kaempferol, quercetin and caffeic acid was high performance liquid chromatography analyses, for tannic acid was Folin-Denis assay, for total polyphenols was Folin-Ciocalteu assay, respectively.

Biochemical measurements

Total cholesterol, triglycerides (TG), glucose, and HDL-cholesterol in serum were measured using a FUJI DRI-CHEM 7000Z analyzer (Fujifilm, Tokyo, Japan). Liver TG and serum GOT and GPT were measured by using enzymatic kits (Triglyceride E test Wako, Transaminase CII Wako; Wako Pure Chemical Industries, Osaka, Japan).

Determination of TG content in the liver and lipid content in feces

Preparation of lipids in the liver was done according to the Folch method (10) with some modification. Samples were homogenized with 10 mL of water. Two mL of the homogenate was mixed with 5 mL of chloroform/methanol (2/1). After vigorously shaking the sample, the tube was centrifuged at 3,000 rpm for 5 min. The lower chloroform phase was collected, and chloroform/methanol was added to the residual sample and centrifuged at 3,000 rpm for 5 min. The lower phase was collected again. The lower chloroform phase mixture containing lipids was filtrated and dried at 80°C to vaporize the solvents, and then the TG content was measured using the Triglyceride E test Wako.

Fecal lipids were obtained by the hot ether extraction method (11).

Indirect calorimetry

Rats were placed in a chamber with food and water, and oxygen consumption and carbon dioxide production were measured automatically for 24 hours at a flow rate of 3 ml / min. Oxygen consumption was continuously measured during the 12-h light-dark cycles using a comprehensive laboratory animal open-circuit indirect calorimetry monitoring system (Columbus Instruments, Columbus, OH, USA).

Quantitative reverse transcribed (RT)-PCR analysis

Total RNA was isolated from the liver using an RNAiso Plus (Takara Bio, Shiga, Japan). First-strand cDNA was reverse-transcribed from the extracted total RNA with PrimeScript™ Master Mix (Takara Bio). Real-time PCR was performed by using specific primers and SYBR green dye (Applied Biosciences, USA) in a StepOne Plus™ real-time PCR system (Applied Biosciences) according to the manufacturer's instructions. The primers used were 5'- GAGATGTGCAAACAGGGCTA -3' (sense) and 5'- CAG TCCTCTCCTCAGTCACG -3' (antisense) for adipose triglyceride lipase (ATGL), 5'- CCAGTCTACATCCGCTTGGCTGAG -3' (sense) and 5'- AGTCGCCAGTAGTAGAAGAAGGTGCGG -3' (antisense) for acetyl CoA carboxylase (ACC), 5'- TGGGCCAG CTTCTTAGCC -3' (sense) and 5'- GGACCAGCGCAGTACCGT AGA -3' (antisense) for fatty acid synthase (FAS), 5'- GGAGCCA TGGATTGCACATTT -3' (sense) and 5'- TCCTTCCGAAGGTC TCTCCTC -3' (antisense) for sterol regulatory element binding protein 1 (SREBP1), and 5'-ACCCTGAAGTACCCCATTTGA -3' (sense) and 5'-GGTGGCCTTAGGGTTCAGAG -3' (antisense) for β -actin.

Statistics

Data were analyzed using unpaired Student's *t* test. Data are expressed as means \pm SD. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of PTL on body weight and fat weight in Wistar rats fed a high-fat diet

At first, we investigated the effects of PTL on body weight and fat weight in rats fed a high-fat diet. Although a statistically significant difference was not observed, body weight in Wistar rats treated with PTL tended to be less than that in control rats during the experimental period (Fig. 1). We determined the amounts of experimental diet intake during experimental period from 1 to 6 weeks, and a difference was not observed between the two groups (Fig. 2). Next, we compared organ weights in the two groups of rats. As shown in Table 2, a difference was not found between the two groups in weight of the liver, kidney, stomach, pancreas and mucosa of the intestine. In visceral fat weight of PTL-treated rats, mesenteric fat was significantly decreased and perirenal and epididymal fats tended to be decreased compare to those in control rats (Table 2).

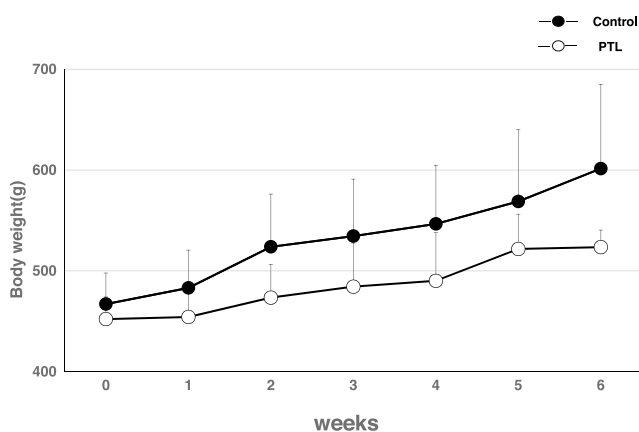


Figure 1. Effect of PTL on body weight gain in Wistar rats fed a high-fat diet. Rats were fed a high-fat diet and treated with PTL (black circle) ($n = 5$) or not treated with PTL (White circle) ($n = 5$). Values are means \pm SD of 5 rats. The data shown were representative of two independent experiments.

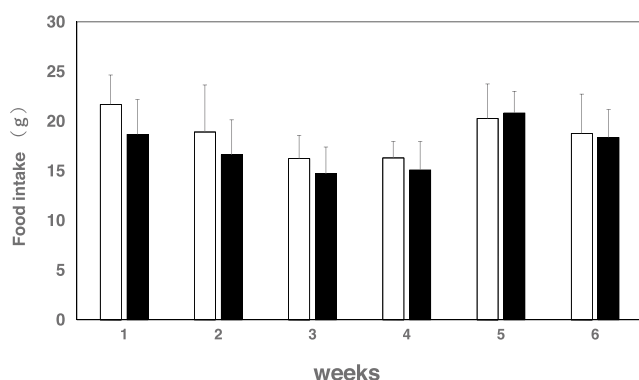


Figure 2. Food intake in rats fed a high-fat diet during 6 weeks. Amount of food intakes in control and PTL groups from 1 to 6 weeks were shown. White bar, high-fat diet (HFD); black bar, high-fat diet plus PTL. Values are means \pm SD of 5 rats. A statistically significant difference was not observed during the experimental period.

Table 2. Organ weight in PTL-treated Wistar rats fed a high-fat diet¹

	Organ weight (g/ 100 g body weight)	
	Control	PTL
Liver	2.29 ± 0.05 ²	2.41 ± 0.10
Stomach	0.44 ± 0.06	0.42 ± 0.05
Pancreas	0.20 ± 0.01	0.16 ± 0.04
Kidney	0.53 ± 0.03	0.55 ± 0.03
Small intestinal mucosa	1.12 ± 0.12	1.13 ± 0.12
Perirenal fat	3.53 ± 0.51	2.75 ± 0.84
Epididymal fat	2.96 ± 0.54	2.65 ± 0.37
Mesenteric fat	1.94 ± 0.25	1.38 ± 0.04*

¹After 6 weeks of the treatment, rats were sacrificed and each organ weight was determined.

²mean ± SD

* $P < 0.05$

PTL improves the serum lipid profile in rats fed a high-fat diet

We compared serum biochemical parameters in the two groups. Although serum levels of glucose, GOT and GPT were not different between the two groups, there were significant difference in the level of TG, total cholesterol and HDL-cholesterol (Table 3). Since we found that PTL regulates serum lipid profiles, we determined TG contents in the liver and fat excretion into feces. Although there were no significant differences, TG contents in the liver tended to be decreased and fat excretion into feces tended to be increased in PTL-treated rats (Table 4). We determined the mRNA expression levels of *ATGL*, *ACC*, *FAS* and *SREBP1*, which contribute to lipid metabolism, by real-time PCR. Relative mRNA expression levels of *ATGL*, *ACC*, *FAS* and *SREBP1* genes were 1.42 ± 0.83 vs 2.10 ± 0.24 ($p = 0.147$), 1.25 ± 0.83 vs 1.94 ± 0.72 ($p = 0.109$), 3.39 ± 1.75 vs 3.42 ± 2.12 ($p = 0.978$), and 1.47 ± 0.51 vs 1.39 ± 0.21 ($p = 0.740$) in the control and PTL groups, respectively.

PTL increases energy metabolism in rats fed a high-fat diet

We found that PTL can regulate serum lipid profiles and attenuates gain of body and fat weights. We assumed that PTL affects metabolism in the body that results in decreases in body and fat weights. We determined energy metabolism in the light and dark periods and found that energy expenditure in PTL-treated rats in the dark period but not light period was significantly higher than that in control rats (Fig. 3).

Table 3. Effect of PTL on biochemical parameters in Wistar rats fed a high-fat diet¹

	Control	PTL
Glucose (mg/dl)	75.8 ± 3.7 ²	76.0 ± 3.2
Triglyceride (mg/dl)	84.8 ± 6.3	66.3 ± 10.8*
Total cholesterol (mg/dl)	66.0 ± 7.3	48.4 ± 11.6*
HDL-cholesterol (mg/dl)	35.7 ± 5.1	47.7 ± 1.9*
GOT (IU/L)	75.4 ± 6.6	64.5 ± 13.6
GPT (IU/L)	10.7 ± 0.9	13.0 ± 2.5

¹After 6 weeks of the treatment, rats were sacrificed and biochemical parameters in serum were determined.

²mean ± SD

* $P < 0.05$

Table 4. Accumulation of triglyceride in the liver and fat excretion into feces in PTL-treated Wistar rats fed a high-fat diet¹

	Control	PTL
Triglyceride (mg/g liver)	68.6 ± 27.2 ²	56.3 ± 16.9
Fecal fat (g/3 day)	0.25 ± 0.03	0.31 ± 0.10

¹After 6 weeks of treatment, rats were sacrificed and triglyceride in the liver were determined. Feces were collected from 5 to 6 weeks in the experiment and fat excretion into feces was determined.

²mean ± SD

DISCUSSION

In this study, we evaluated the food functionality of dietary PTL in an animal model of diet-induced obesity. We found that PTL regulates lipid metabolism including TG, total cholesterol and HDL-cholesterol. To our knowledge, this is the first study showing that PTL regulates lipid metabolism *in vivo*.

Polyphenols have been shown to possess a variety of biological functions. Animal and human studies have suggested that polyphenols derived from plants have beneficial effects for diseases. PTL contains various kinds of polyphenols. PTL contains about 2% of polyphenols and the major components are shown in Table 1. The most abundant polyphenol component is tannic acid. Although we investigated whether tannic acid can change serum lipid levels by replacing dietary PTL with tannic acid, serum lipid levels were not changed in high-fat diet-treated rats (data

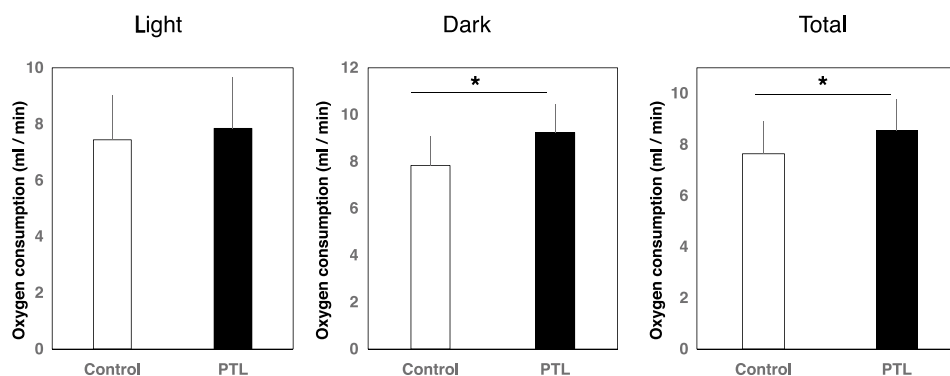


Figure 3. PTL increases energy expenditure in the dark period but not in the light period. Energy expenditure was determined in dark and light periods as described in the MATERIALS AND METHODS section. White bar, high-fat diet (HFD); black bar, high-fat diet plus PTL. Values are means ± SD of 5 rats. * $P < 0.05$. The data shown were representative of two independent experiments.

not shown). The results indicate that tannic acid does not directly affect lipid metabolism. In this section, we discuss the contribution of three other major polyphenols and biological markers obtained from our results.

Kaempferol is a natural flavonol-type flavonoid and can be isolated from tea as well as numerous common vegetables and fruits (12). The most well-known properties of kaempferol are its anti-inflammatory effects (12). Some studies have the effects of kaempferol on focused on adipocyte differentiation *in vitro* and an animal obesity model *in vivo*. In an *in vitro* study, kaempferol inhibited adipogenesis and suppressed lipid accumulation in 3T3-L1 cells at concentrations of 7.5 to 60 μ M (13, 14). Kaempferol has also been shown to increase oxygen consumption by 30% in normal human skeletal muscle myocytes (15). In an *in vivo* study, treatment with kaempferol at doses of 5 mg and 15 mg/kg body weight decreased body weight gain and visceral fat weight in mice fed a high-fat diet (16). The dose of kaempferol used in this study was estimated to be less than 1 mg/kg body weight.

Tryptanthrin is a natural alkaloid compound that has a basic indoloquinazoline moiety. Similar to the function of kaempferol, tryptanthrin has been shown to have an anti-inflammatory effect. Anti-inflammatory effects of tryptanthrin have been shown in lipopolysaccharide-stimulated microglial cells, human neutrophils and a mouse model of dextran sodium sulfate-induced colitis (17-19). The anti-inflammatory effect of tryptanthrin is partly due to inhibition of prostaglandin and leukotriene synthesis (20, 21). Inflammation is considered to have a pivotal role in the development of metabolic disease. In particular, adipose tissue shows the hallmarks of chronic inflammation. Although an obese status might contribute to inflammation, an anti-obesity effect of tryptanthrin has not been reported.

Indirubin, a traditional Chinese medicine formulation from the Muricidase family, has been reported to exhibit broad anti-cancer and anti-inflammation activities (22, 23). By using a cell-based approach to screen a library of pharmacologically active small molecules, indirubin-3'-oxime was shown to be a Wnt/ β -catenin activator (24). Indirubin-3'-oxime inhibited the differentiation of 3T3-L1 cells into mature adipocytes and decreased the expression of adipocyte markers. In an *in vivo* study by Choi et al., indirubin-3'-oxime inhibited the development of obesity in high-fat diet-fed mice by attenuating body weight gain and visceral fat accumulation (25). In that study, treatment with indirubin-3'-oxime at doses of 25-100 mg/kg body weight decreased serum TG and total cholesterol contents (25).

We discussed the regulation of lipids by PTL from the viewpoint of polyphenol functions. Other ingredients in PTL should be considered. However, we cannot discuss the effects of other ingredients here due to the lack of information about ingredients of PTL.

One of the limitations of this study is the large variation of data. Significant differences were not observed in body weight, energy expenditure in the light period, and perirenal and epididymal fat weights. However, these values tended to be decreased in the PTL-treated group. Further study is needed to obtain reliable results and elucidate the mechanism for improvement of lipid profiles by PTL.

We were not able to determine what ingredient is effective for improving lipid profiles by PTL. However, we found that PTL has a beneficial effect in rats fed a high-fat diet. Our finding raises the possibility that PTL is applicable for a food supplement.

CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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