ORYZA OIL & FAT CHEMICAL CO., LTD.

WALNUT POLYPHENOL

Hepatoprotective & Anti-oxidative Extract For Metabolic Syndrome

- **WALNUT POLYPHENOL-P10**
  (Powder, Food Grade)
- **WALNUT POLYPHENOL-WSP10**
  (Water-soluble Powder, Food Grade)
- **WALNUT POLYPHENOL-PC10**
  (Powder, Cosmetic Grade)
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  (Water-soluble Powder, Cosmetic Grade)
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  (Water-soluble Liquid, Cosmetic Grade)
- **WALNUT SEED OIL**
  (Oil, Food)

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1. Introduction

Recently, there is an increased awareness on metabolic syndrome – a condition characterized by a group of metabolic risk factors in one person. They include abdominal obesity, atherogenic dyslipidemia, elevated blood pressure, insulin resistance, prothrombotic state & proinflammatory state. The dominant underlying risk factors appear to be abdominal obesity and insulin resistance. In addition, non-alcoholic fatty liver disease (NAFLD) is the most commonly associated "liver" manifestation of metabolic syndrome which can progress to advance liver disease (e.g. cirrhosis) with associated morbidity and mortality. Lifestyle therapies such as weight loss significantly improve all aspects of metabolic syndrome, as well as reducing progression of NAFLD and cardiovascular mortality.

Walnut (*Juglans regia* L. seed) is one the most popular nuts consumed in the world. It is loaded in polyunsaturated fatty acids – linoleic acid (LA), oleic acid and α-linolenic acid (ALA), an ω3 fatty acid. It has been used since ancient times and epidemiological studies have revealed that incorporating walnuts in a healthy diet reduces the risk of cardiovascular diseases. Recent investigations reported that walnut diet improves the function of blood vessels and lower serum cholesterol. Nevertheless, walnut is rich in micronutrients (vitamins & minerals), plant sterols and polyphenols.

A joint research project was conducted between Oryza Oil & Fat Chemical Co., Ltd. and Pola Chemical Industries, Inc. to study the physiological effects of testa of walnut. Various hydrolysable polyphenolic compounds were identified which are potent antioxidants and liver protective. Correspondingly, Walnut Polyphenol is believed to be beneficial in the treatment of metabolic syndrome.

Fig. 1. Walnuts (*Juglans regia* L.)

2. WALNUT POLYPHENOLS

Most polyphenolic compounds of walnut are loaded in the testa of walnut fruit as illustrated in Fig. 2. These are hydrolysable polyphenolic compounds as shown in Fig. 3 and its principle constituents are as listed in Table 1.

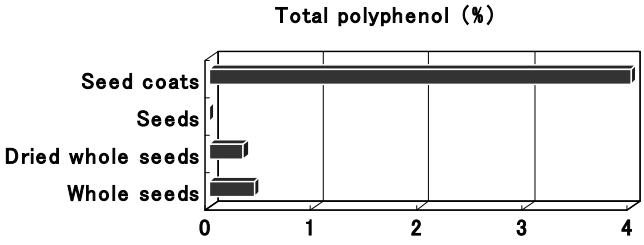


Fig. 2. The Content of Polyphenols in Walnut (%)
（Fukuda T. Polyphenols from walnuts: Structures and functions. IFT 2006 Annual Meeting, Orland, Florida, June 24-28, 2006）

Table 1. Principle Polyphenolic Constituents and Their Respective Distribution in Walnut

	(%)
Pedunculagin (**1**)	16.0
Ellagic acid (**5**)	15.8
Tellimagrandin I (**6**)	6.6
Casuarictin (**7**)	4.1
Tellimagranin II (**10**)	1.2
Rugosin C (**11**)	1.8
Casuarinin (**12**)	1.0

Fukuda T. *et al*., *BioFactors*, **21**, 251-253 (2004).

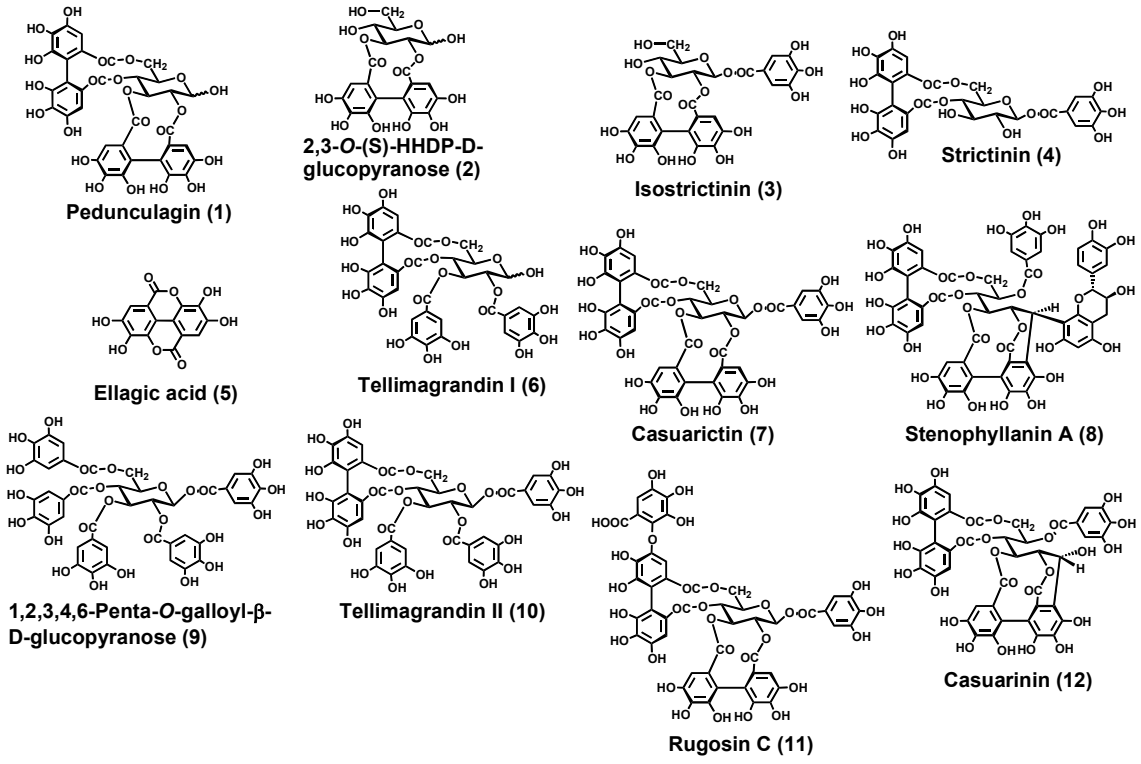
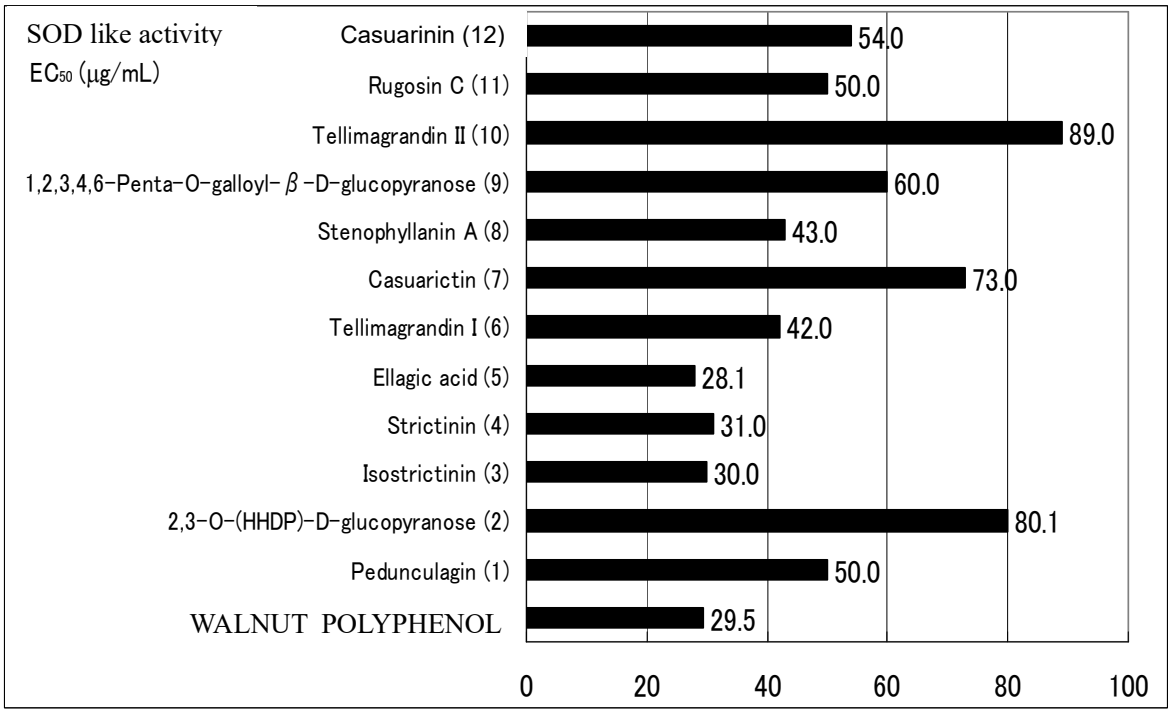


Fig. 3. Chemical Structures of Hydrolysable Polyhenolic Compounds of Walnut



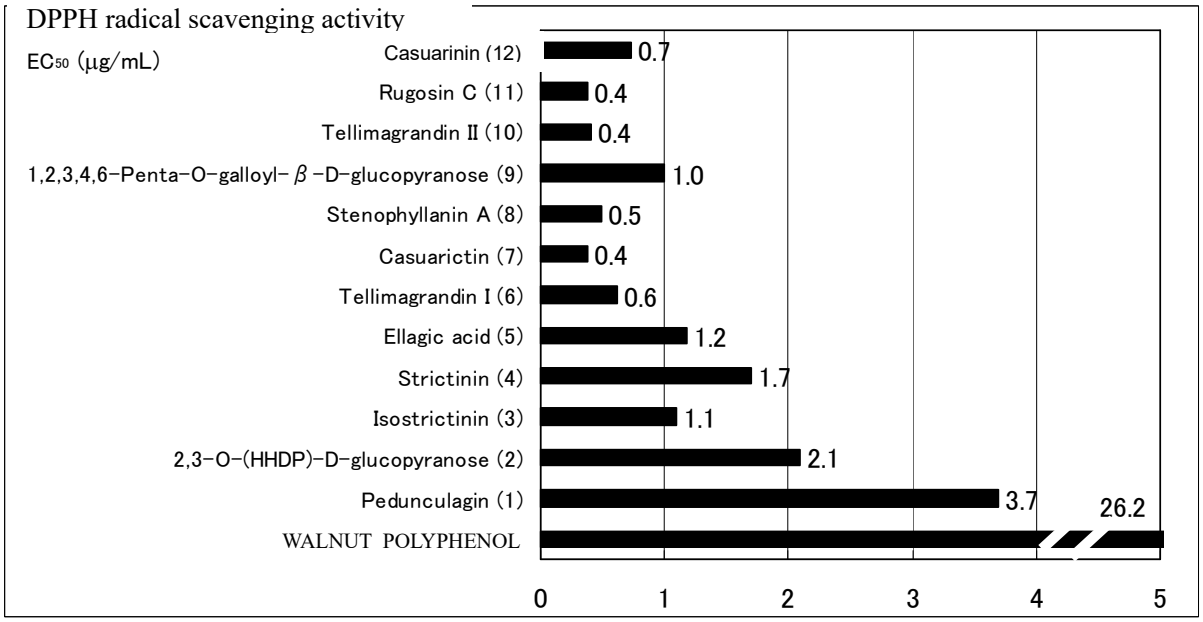


Fig. 4. (top) SOD Antioxidant Activity of Walnut Polyphenols
(bottom) DPPH Antioxidant Activity of Walnut Polyphenols

Fukuda T., *et al.*, Antioxidative polyphenols from walnuts (*Juglans regia* L.). *Phytochemistry*, **63**, 795-801 (2003).

EC_{50}: 50% effective concentration of sample

3. Hepatoprotective Effect of WALNUT POLYPHENOLS

(I) *In vivo*

Liver is the second largest organ in the human body. It plays a major role in metabolism of human body including glycogen storage, plasma protein synthesis and drug detoxification. It regulates a wide variety of high volume biochemical reactions and breaks down toxic substances (e.g. chemicals, viruses, alcohol etc) which may result in toxication (Fig. 5).

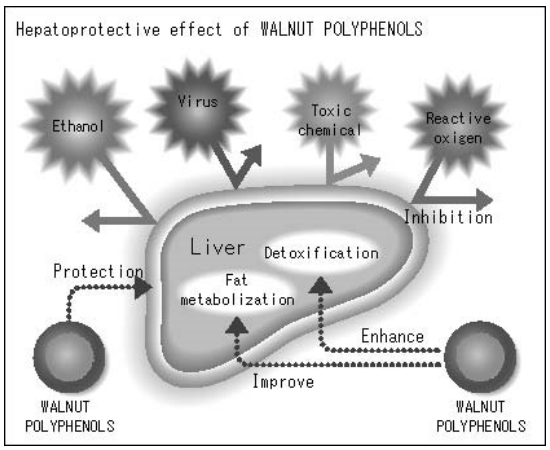


Fig. 5. Liver Functions and The Effect of Walnut Polyphenols on Liver Health

(a) The effect of Walnut Polyphenols on CCl_4-induced liver damage in mice model (detoxification)

The effect of Walnut Polyphenols on CCl_4-induced liver damage was studied. **This model is a simulated human hepatitis model as a result of oxidative stress and toxication.** Upon liver damage, the liver enzymes GOT (glutamate oxaloacetate transaminase) and GPT (glutamic pyruvic transaminase) infiltrate into the blood stream resulting in elevated serum GOT & GPT levels. Walnut Polyphenols demonstrated a dose dependent lowering effect in GOT & GPT in CCl_4-induced liver damage in mice model (Fig. 6). Meanwhile, Walnut Polyphenols is more superior than Curcumin, a commonly used hepatoprotective agent.

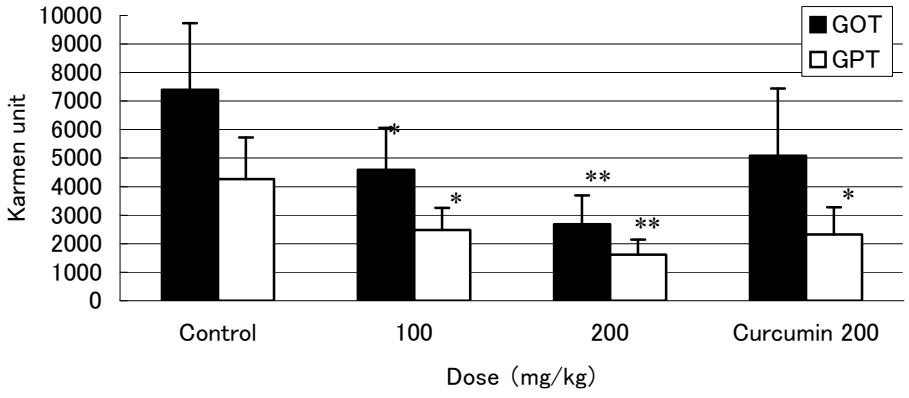


Fig. 6. The Effect of Walnut Polyphenols on CCl_4-induced liver damage in mice model（n=8, mean±S.E., *: p<0.05, **: p<0.01）

【Method of Experiment】

Fasting mice (ddY, male, 5-wk old) were orally fed Walnut Polyphenols. One hour later, back of mice were subcutaneously injected with 10% CCl_4 previously diluted in olive oil (5ml/kg). Blood samples were collected 20 hours later for measurement of serum GOT and GPT.

(b) The Effect of Walnut Polyphenols on D-galactosamine–induced liver damage in mice (detoxification)

The effect of Walnut Polyphenols on D-galactosamine (D-GaIN)–induced liver damage was studied. This is a simulated human viral hepatitis model based on immune responses and histological observations. As illustrated in Fig. 7, both GOT and GPT levels are elevated in the control group 10-hour post induction of D-GaIN. Meanwhile, group treated with Walnut Polyphenols demonstrated a dose-dependent reduction on GPT level although no changes observed in the GOT level.

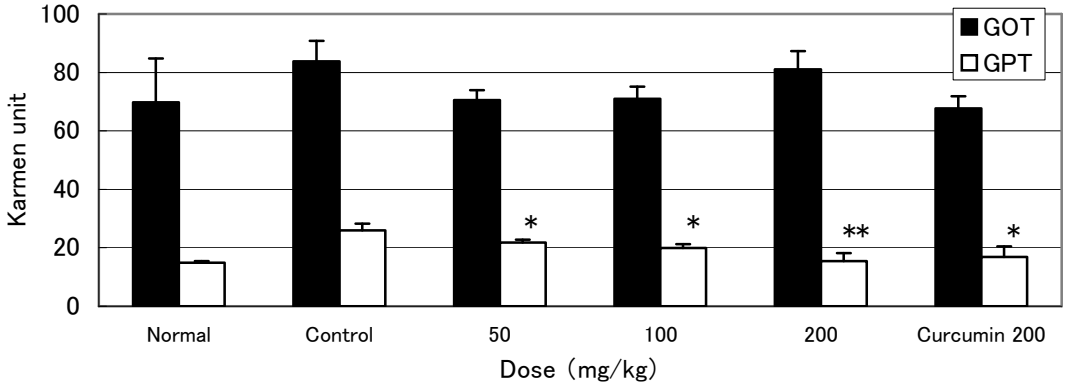


Fig. 7. The effect of Walnut Polyphenols on D-Galactosamine–induced liver damage in mice. (n=8, mean±S.E., *: $p<0.05$, **: $p<0.01$)

【Method of Experiment】

Fasting mice (ddY, male, 5-wk old) were orally fed Walnut Polyphenols followed by intra-peritoneal injection of D-galactosamine (300mg/kg) 1 hour later. Blood samples were collected 20 hours later for measurement of serum GOT and GPT.

(c) The Effect of Walnut Polyphenols on Ethanol–induced liver damage in mice

Ingestion of large amount of ethanol induce GOT and GPT elevations and decrease glutathione (GSH) in rats. [Borknt S. *et al. World Gastroenterol.* **12**, 4345 (2006).] We evaluated the effect of Walnut Polyphenols on the model. As a result, Walnut Polyphenols suppressed GOT and GPT elevations, and the GSH was elevated (Table 2). Walnut Polyohenol is suggested to be effective for liver damage by ethanol.

Table 2. The effect of Walnut Polyphenols on Ethanol–induced liver damage in rats.

	N	GOT (K. unit)	GPT (K.unit)	GSH (μM)
Non-treated	5	62.2±9.2	12.4±1.3	757±42
Control	5	74.9±7.2	15.3±2.0	743±19
Walnut Polyphenols 100 mg/kg	6	62.8±10.2	13.1±2.3	777±22

mean±S.E.

【Method of Experiment】

Fasting rats (Wistar, male, 8-wk old) were orally fed Walnut Polyphenols followed by oral administration of ethanol (10 ml/kg) 1 hour later. Blood samples were collected 2 hours later for measurement of serum GOT, GPT and GSH.

(II) *In vitro*

The Effect of Walnut Polyphenols on CCl_4-and D-GalN induced cytotoxicity in rat hepatocytes

The effect of each active component of Walnut Polyphenols on CCl_4 and D-GalN–induced cytotoxicity in primary cultured rat hepatocytes was evaluated *in vitro*. Results showed that tellimagrandin I (**6**), casuarictin (**7**), tellimagrandin II (**10**), and rugosin C (**11**) are inhibitory on CCl_4-induced cytotoxicity in primary cultured rat hepatocytes (as tabulated in Table. 3). Meanwhile, tellimagrandin I (**6**) of Walnut Polyphenols is believed to be the most important active responsible for hepatoprotective effect. Nevertheless, tellimagradin I (**6**) is the third largest component found within Walnut Polyphenols (Table 1).

On the other hand, many constituents including tellimagrandin I (**6**) and 2,3-*O*-(S)-HHDP-D-glucopyranoside (**2**) suppressed cytotoxicities. Fig. 8 shows structure-activity relation ship of walnut polyphenol. In CCl_4-induced cytotoxicity, galloyl glucose is essential for the activity, and 2,3-*O*-(S)-HHDP-D-glucopyranoside (**2**) is minimum structure for the activity in D-Gal-induced cytotoxicity.

【Method of Experiment】

Rat primary cultured hepatocytes (4×10^4 cells/100 μL) were cultured (4 hr) and the medium was chaged to new one containing 5 mM CCl_4 or 10 mM D-GalN and samples. After 40 hr culture, cytotoxicity was evaluated by MTT assay.

Table 3. Effect of constituents of Walnut Polyphenols on CCl_4- and D-GalN-induced hepatocyte damages.

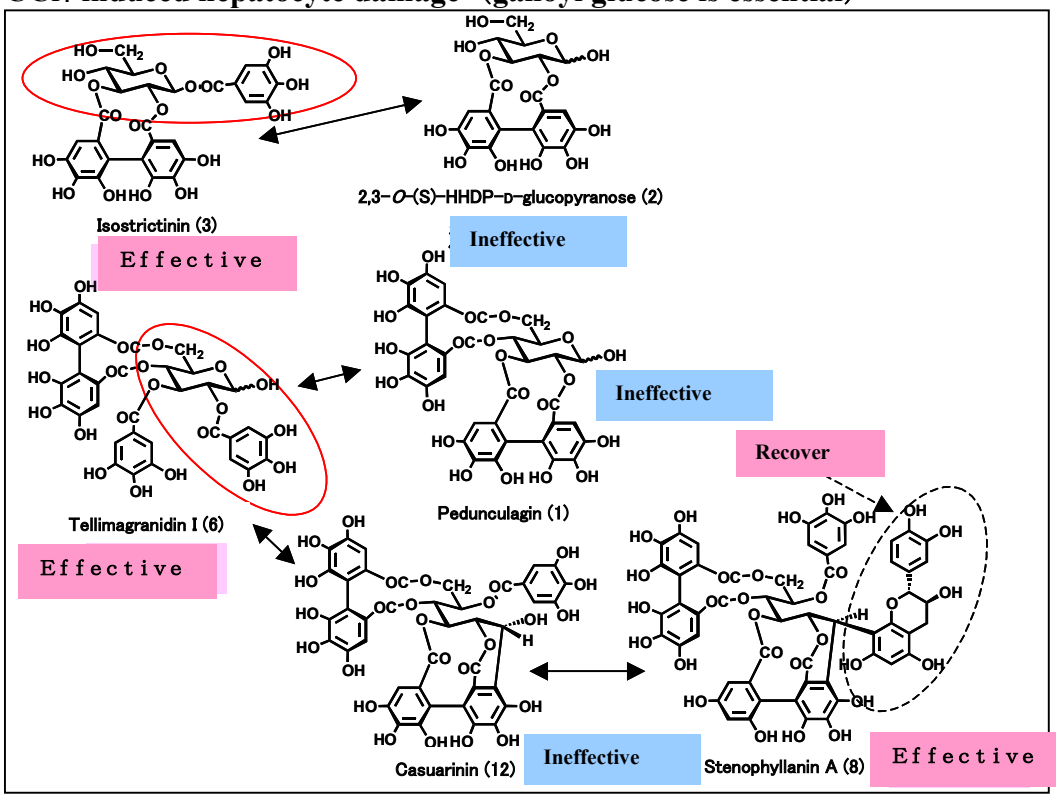
CCl_4	Inhibition (%)		
	10（μg/mL）	30	100
Walnut Polyphenol	7.6±0.2	13.2±0.3	**42.1±1.8****
Pedunculagin (**1**)	4.7±0.2	3.4±0.1	15.7±0.7*
Tellimagrandin I (**6**)	**66.7±9.4***	**53.6±2.8****	**100.0±6.7****
Casuarinin (**12**)	3.1±0.1	8.9±0.2	12.0±0.6
Rugosin C (**11**)	15.8±0.4	36.0±1.4**	**58.8±0.7****
Casuarictin (**7**)	12.6±0.0.2	13.9±0.8	**48.3±1.9****
Tellimagrandin II (**10**)	35.5±3.2	20.4±1.2	**54.8±4.6****
Ellagic acid (**5**)	-12.6±0.5	-18.5±-1.3	-
Strictinin (**4**)	7.3±0.2	12.9±0.9**	**30.9±0.6****
Stenophyllanin (**8**)	7.5±0.2	27.2±0.9**	**37.8±1.2****
Isostrictinin (**3**)	5.1±0.1	5.9±0.3	24.0±1.1**
2	-10.6±0.3	4.7±0.3	4.1±0.1
9	6.6±0.4	22.8±0.5**	27.1±0.6**
Gallic acid	19.5±1.6	28.5±1.5	21.5±1.2
Curcumin	8.5±0.3	5.9±0.1	-

D-GalN	Inhibition (%)		
	10（μg/mL）	30	100
Walnut Polyphenol	**34.0±2.1***	**92.5±8.2****	**104.0±4.9****
Pedunculagin (**1**)	19.0±0.6	11.8±0.8	6.5±0.5
Tellimagrandin I (**6**)	2.3±0.1	**42.5±1.3***	**100.0±3.5****
Casuarinin (**12**)	16.8±0.5	9.3±0.4	10.2±0.7
Rugosin C (**11**)	11.3±0.6	2.8±0.1	29.2±2.8
Casuarictin (**7**)	-5.4±0.2	-0.5±0.1	**86.7±4.1****
Tellimagrandin II (**10**)	4.4±0.1	4.4±0.3	**80.2±2.8****
Ellagic acid (**5**)	4.3±0.7	19.5±0.5	16.5±0.3
Strictinin (**4**)	**56.9±5.7**	**82.3±5.9**	**94.1±2.8**
Stenophyllanin (**8**)	**36.4±2.4**	**92.4±4.9** **	**83.3±3.8** *
Isostrictinin (**3**)	13.8±1.0	**42.5±0.8**	**80.0±5.1**
2	15.8±0.8	**92.0±4.0**	**121.1±6.2** *
9	-16.6±1.9	**37.0±1.8**	**80.2±8.6**
Gallic acid	**60.5±4.3****	**63.6±2.3****	**58.2±4.3****
Curcumin	0.0±0.1	**33.3±1.2**	**47.7±2.0***

Mean±S.E. (n=6-8), *: $p<0.05$, **: $p<0.01$.

Shimoda H., Tanaka J., Kikuchi M., Fukuda T., Ito H., Hatano T., Yoshida T. Walnut polyphenols prevent liver damage induced by carbon tetrachloride and D-galactosamine: hepatoprotective hydrolyzable tannins in the kernel pellicles of walnut. *J. Agric. Food Chem.* **56**, 4444-9. (2008).

CCl_4-induced hepatocyte damage（galloyl glucose is essential）



D-GalN-induced hepatocyte damage (○: compound 2 is minimum, essential moiety. Additional adhesion of HHDP diminish the activity and adhesion of galloyl moiety buck up the activity.)

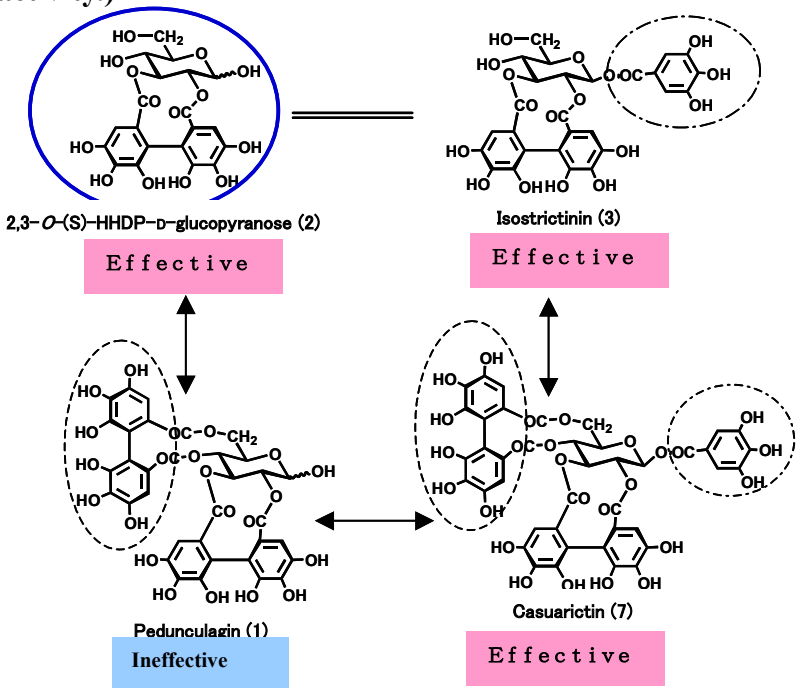


Fig. 8. Structure activity relation ship of Walnut Polyphenols and hepatoprotection.

4. The Effect of Walnut on Metabolic Syndrome

(I) The Effect of Walnut on Atherosclerosis

Epidemiological studies have associated nut consumption with a reduced incidence of cardiovascular mortality. Endothelial dysfunction is associated with atherosclerosis and its risk factors, including hypercholesterolemia. Ros E. *et al*. reported that substituting walnuts for monounsaturated fat in a Mediterranean diet improves endothelium-dependent vasodilation (EDV) in hypercholesterolemic subjects. As illustrated in Fig. 9, daily intake of 8-13 walnuts for 4 weeks significantly improve the EDV of 21 hypercholesterolemic males and females.

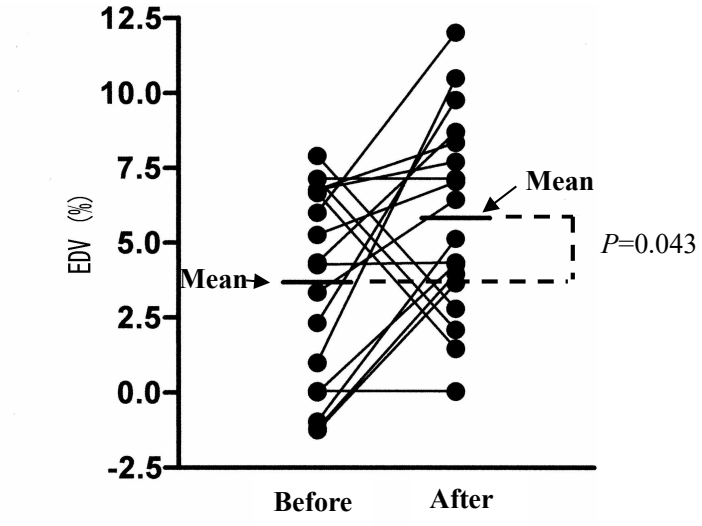
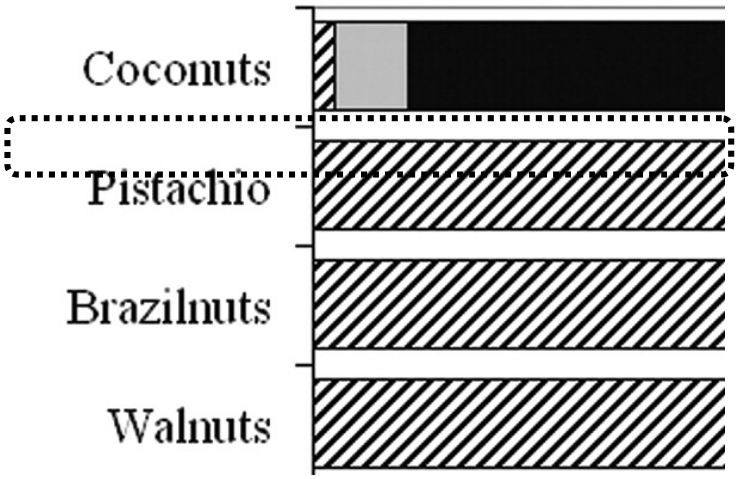


Fig. 9. The effect of Walnut enriched diet on endothelium-dependent vasodilation (EDV) of hypercholesterolemic patients.

Ros E. *et al*., A walnut diet improves endothelial functions in hypercholesterolemic subject. *Circulation*, **109**, 1609-1614 (2004).



Fatty acid composition in walnut oil

	(%)
Palmitate	6.1
Stearate	2.9
Oleate	15.1
Linoleate	58.6
α -linolenate	16.9

Fig. 10. The Fatty Acids Composition of various nuts (PUFA: polyunsaturated fatty acid, MUFA: monounsaturated fatty acid, SFA: saturated fatty acid), Right figure, Fatty acid composition in walnut oil (Left, table).

Mukuddem-Petersen J., *et al*., A systematic review of the effects of nuts on blood lipid profiles in humans. *J. Nutr*. **135**, 2082-2089 (2005).

Fig. 10 illustrated the fatty acids composition of various nuts commonly available in our diet. Walnuts, differ from other nuts, are loaded with α-linolenic acid, a plant ω-3 fatty acids, which may have protective effect on cardiovascular health by reducing blood cholesterol and triglyceride levels.

(II) The Effect of Walnut on Cholesterol

There are several reports documented the beneficial effect of walnuts on hyperlipidemia. [Mukuddem-Petersen J., *et al*., *J. Nutr*. **135**, 2082-2089 (2005)] In one study conducted by Iwamoto M *et al*. on Japanese subjects are particularly interesting. Iwamoto M *et al.*, reported that incorporating moderate quantities of walnuts into the average Japanese diet while maintaining the intake of total dietary fat and energy decreases serum total cholesterol concentration and favorably modifies the lipoprotein profile in Japanese, particularly in women. In this study, daily intake of 43 to 57g of walnuts was incorporated into Japanese diet for 4 weeks to 40 healthy Japanese men and women. As illustrated in Fig. 11, blood cholesterol of test subjects was lowered, particularly in women.

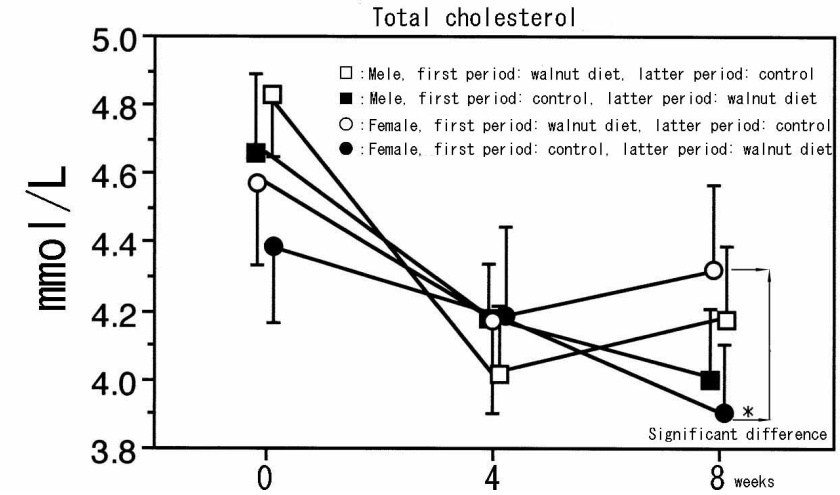
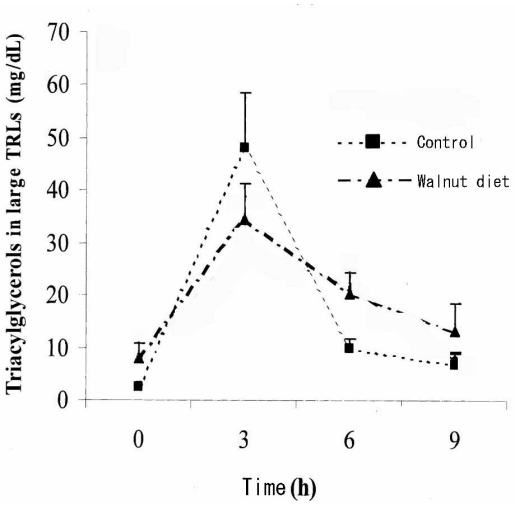


Fig. 11. The effect of Walnuts on Total Cholesterol (*: p<0.05)
Iwamoto M. *et al*., Walnuts lower serum cholesterol in Japanese men and women. *J. Nutr.* **130**, 171-176 (2000).



On the other hand, Bellido C.E. *et al.*, reported that walnut-enriched meals effectively prevented post prandial lipidemia where triacylglycerol in large triacylglycerol is significantly reduced.

Fig. 12. The Effect of Walnuts on postprandial blood triacylglycerol.
Bellido C. E. *et al*., Butter and walnuts, but not olive oil, elicit postprandial activation of nuclear transcription factor κB in peripheral blood mononuclear cells from healthy men. *Am. J. Clin. Nutr.*, **80**, 1487-1491 (2004).

(III) Effect of Walnut Polyophenol on hypercholesterolemia

We investigated the effect of Walnut Polyphenol on hypercholesterolemia in mice fed high cholesterol diet and high fat diet. By 6-day oral treatment of Walnut Polyphenol (200 mg/kg), serum and liver cholesterol were decreased in high cholesterol diet fed rats (Table 4). Walnut Polyphenol was found to decrease cholesterol level in diet-induced hypercholesterolemia.

Table 4. Effect of Walnut Polyphenol on serum and liver cholesterol elevation in high cholesterol diet-fed mice.

	Normal diet	Control	Walnut Polyphenol
Serum cholesterol （mg/dL）	141±7**	365±40	226±27**
Liver cholesterol (mg/g)	4.7±1.1*	8.1±0.8	5.9±0.8

Mean±S.E.(N=7), *: $p < 0.05$, **: $p < 0.01$

【Method of Experiment】

Mice (ddY, male, 5-wk) were fed high cholesterol diet for 6 days and Walnut Polyphenol (200 mg/kg) was given orally once a day. Mice were fasted during 6 to 7 day and blood and liver were collected.

(IV) Effect of Walnut Polyophenol on hypertriglychemia and fatty liver

We investigated the effect of Walnut Polyphenol on high fat diet –fed mice. High fat diet was fed to mice for 2 weeks and Walnut Polyphenol was given orally once a day. As shown in Table 5, Walnut Polyphenol reduced increase in body weight, liver weight, liver triglyceride and serum triglyceride.

【Method of Experiment】

Mice (ddY, male, 10-wk) were fed high fat diet for 2 weeks and Walnut Polyphenol was given orally once a day. Then mice were fasted and organ and blood were collected.

Shimoda H., Tanaka J., Kikuchi M., Fukuda T., Ito H., Hatano T., Yoshida T. Effect of polyphenol-rich extract from walnut on diet-induced hypertriglyceridemia in mice via enhancement of fatty acid oxidation in the liver. *J. Agric. Food Chem.* **57**, 1786-92. (2009).

Table 5. Effect of Walnut Polyphenol on lipid parameters in mice fed high fat diet (HDF)

		Control	Walnut Polyphenol		
	Normal diet	HDF	50 (mg/kg)	100	200
Body weight					
Initial (g)	40.2±0.2	40.6±0.7	40.0±0.6	39.4±0.3	38.7±0.3
13 day (g)	43.9±0.4	47.7±1.4	45.2±1.2	44.7±0.6	44.0±1.4
Increase (g)	3.7±0.5	7.0±0.9	5.3±0.9	5.2±0.7	5.3±1.4
Organ weight					
Liver (g)	1.56±0.03	1.60±0.08	1.44±0.07	1.41±0.03*	1.34±0.03**
Peri-renlal fat (mg)	261±48**	736±88	756±88	729±106	773±161
Epidydimal fat (mg)	732±92**	1814±282	1817±188	1768±150	1637±227
Liver lipid					
Triglyceride (mg/g)	21.7±3.4	31.8±3.9	32.9±4.8	25.6±3.2	25.8±5.5
Cholesterol (mg/g)	4.5±0.7	6.2±0.9	8.0±1.5	5.6±0.9	6.0±1.2
Serum lipid					
Triglyceride (mg/dL)	148±10	181±21	98±34**	82±6**	98±13**
Cholesterol (mg/dL)	165±5*	218±14	225±12	220±14	220±18
Blood sugar (mg/dL)	113±14**	167±11	177±10	193±11	130±17

Mean±S.E. (N=7), *: $p < 0.05$, **: $p < 0.01$

(V) Effect of Walnut Polyphenol on lipid metabolism in liver

As Walnut Poluphenol did not suppress intestinal lipid absorption and fat accumulation, we evaluated on beta-oxidation in mouse liver fed high fat diet. Liver homogenate was divided into mitochondrial and cytosolic fractions by centrifugation and following reaction was induced using palmitoyl CoA and NAD.

$$CH_3(CH_2)_xCO\text{-}SCoA + O_2 + NAD + CoA \rightarrow$$
$$CH_3(CH_2)_{x-2}CO\text{-}SCoA + H_2O_2 + NADH + H^+ + \text{acetyl-CoA}$$

According to the change of absorbance at 340 nm caused by reduction of NAD, Walnut Polyphenol did not suppressed mitochondrial beta-oxidation, however it tended to enhance cytosolic beta-oxidation (Table 6). Walnut Polyphenol was found to enhance cytosolic beta-oxidation including microsome.

Table 6. Effect of Walnut Polyphenol on beta-oxidation in mouse liver fed high fat diet

	Dose	Change in absorbance at 340 nm (⊿OD/mg/min)	
	(mg/kg)	Mitochondrial fraction	Cytosolic fraction
Normal diet	-	0.345±0.045	0.0170±0.0019
High fat diet (control)	-	0.320±0.072	0.0093±0.0008
Walnut Polyphenol	50	0.273±0.033	0.0119±0.0025
	100	0.227±0.006	0.0112±0.0024
	200	0.263±0.021	0.0133±0.0030

Mean±S.E. (N=5-7)

In addition, by the investigation of hepatic mRNA expression related to lipid metabolism, expression of PPARalpha and acyl CoA oxidase (ACOX1) were enhanced (Fig. 13). On the other hand, carnitinpalmitoyl transferase (CPT)1A, a rate-limiting enzyme on mitochondrial beta-oxidation did not enhanced. Therefore Walnut Polyphenol was found to enhance cytosolic

beta-oxidation via PPARα.

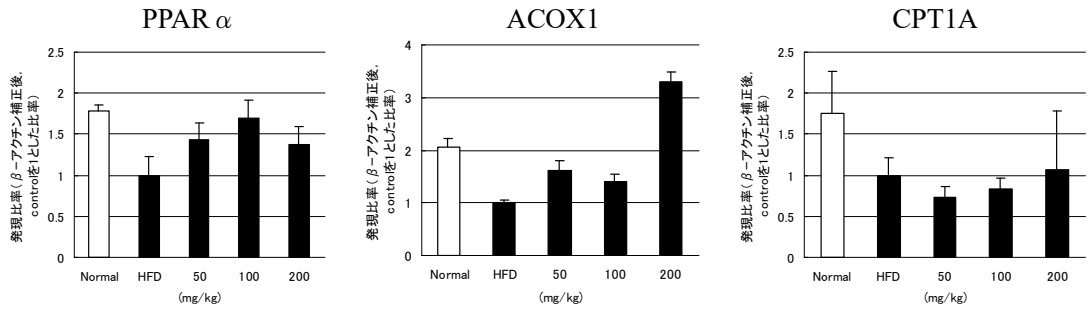


Fig. 13. Effect of Walnut Polyphenol on mRNA expression related to lipid metabolism in mice fed high fat diet.
Mean±S.E (N=5-7)

These results implicate that Walnut Polyphenol reduce serum and liver triglyceride by enhance liver cytosolic beta-oxidation.

（VI）The effect of Walnut Polyphenols on Diabetes Mellitus

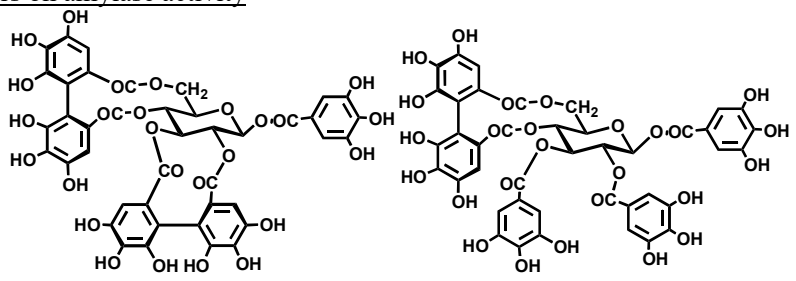
Dr. Fukuda of Pola Chemical Industries, Inc. has conducted extensive studies on the effect of Walnut Polyphenols on diabetes mellitus especially on the enzyme glycosidases. The IC_{50} of each active component of Walnut Polyphenols on glycosidases is tabulated in Table 3. Walnut extract and its polyphenolic components, especially casuarictin (**7**) and tellimagradin II (**10**) demonstrated strong inhibitory activity against amylase. Meanwhile, tellimagradin I (**6**), tellimagradin II (**10**) & casuarictin (**7**) are three most potent walnut polyphenolic components against maltase activity. Walnut Extract and its polyphenolic components showed similar inhibition against the enzyme sucrase.

Table 7 IC_{50} of Walnut Polyphenols on glycosidases

	Inhibitory activity : IC_{50} (mg/mL)		
	Sucrase	maltase	Amylase
Pedunculadin (**1**)	0.50	0.70	0.11
2,3-HHDP-Glc. (**2**)	0.67	0.83	0.13
Isostrictinin (**3**)	0.41	0.31	0.062
Strictinin (**4**)	0.26	0.20	0.053
Tellimagrandin I (**6**)	0.33	**0.041**	0.013
Casuarictin (**7**)	0.30	0.18	**0.0033**
Stenophyllanin A (**8**)	0.92	0.31	Not examined
Tellimagrandin II (**10**)	0.43	**0.025**	**0.0019**
Rugosin C (**11**)	0.60	0.32	0.017
Casuarinin (**12**)	0.40	**0.046**	0.018
Walnut extract	>1	0.40	0.070
non resin-attached fraction	>1	>1	>1
resin-attached fraction	0.61	0.11	0.011

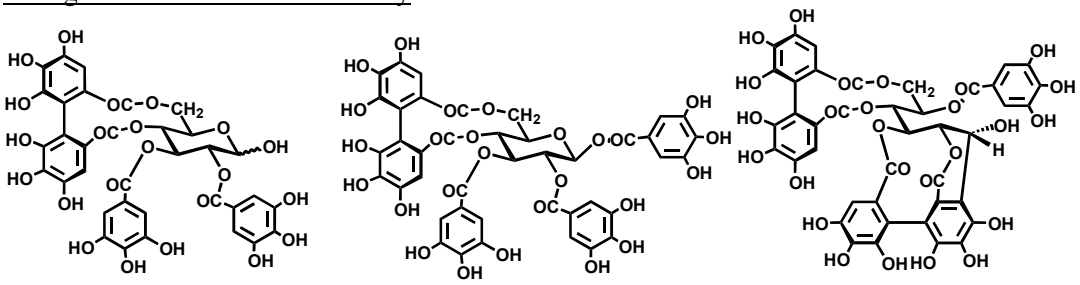
Fukuda, *et al.*, the 50th Meeting of the Japanese Society of Pharmacognosy, Sep. 12-13, 2003 (Tokyo), the 51st Meeting of the Japanese Society of Pharmacognosy, Sep. 9-10, 2004 (Kobe).

Strong inhibitors on amylase activity



Casuarictin (7) **Tellimagrandin II (10)**

Strong inhibitors on maltase activity



Tellimagrandin I (6) **Tellimagrandin II (10)** **Casuarinin (12)**

In a research conducted by Fukuda *et al*., reported that walnut polyphenol-rich fraction (WPF) lowered elevated blood glucose level post-loading of starch and sucrose in mice model (Fig. 14).

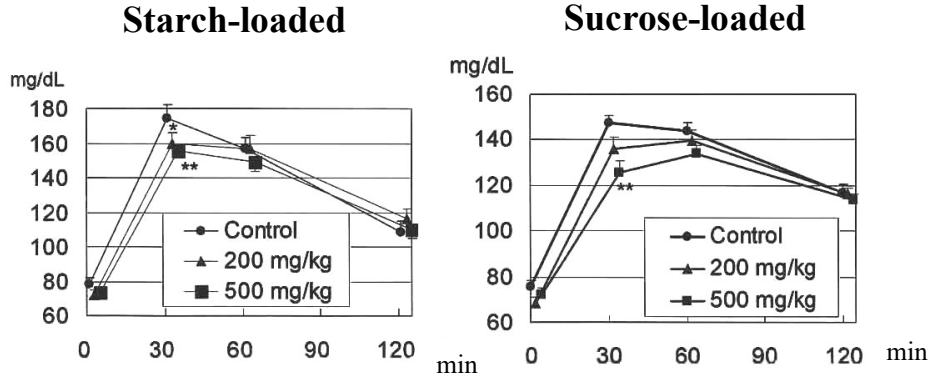


Fig. 14. The effect of Walnut Polyphenol-rich Fraction (WPF) on Blood Glucose Level Post-Loading of Starch and Sucrose in Mice (mean ± S.E., n=23, *: $p<0.05$, **: $p<0.01$)

Fukuda et al., The 50th Meeting of the Japanese Society of Pharmacognosy, Sep 12-13, 2003 (Tokyo), The 51st Meeting of the Japanese Society of Pharmacognosy, Sep 9-10, 2004 (Kobe).

[Method of Experiment]

Blood sample was collected from fasting mice (ddY, male, 10-week old) for measurement of initial blood glucose level prior to loading of starch and sucrose. Test sample A (containing Walnut polyphenol-rich fraction [WPF] and soluble starch 2g/kg)

and test sample B (containing Walnut polyphenol-rich fraction [WPF] and sucrose 2g/kg) were orally given to mice 20 minutes later. Blood samples were collected at 30, 60, and 120 minutes for measurement of blood glucose level.

In addition to the above findings, research also noticed that Walnut polyphenol-rich fraction (WPF) has triglyceride lowering effect and urine peroxide lowering effect in genetically inherited Type II diabetes mellitus (*db*/*db*) mice as shown in Table 4.

Table 4. The effects of walnut polyphenol-rich fraction (WPF) on genetically inherited type II diabetes mellitus mice.

	Genotype	Glucose (mg/dL)	Cholesterol (mg/dL)	Triglyceride (mg/dL)	Urine 8-OHdG/creatinine (ng/mg creatinine)
Normal	*db*/+m	25.6±0.8**	63.5±3.2**	69.3±9.3**	84.0±12.4**
Control	*db*/*db*	37.4±2.4	103.8±19.1	177.0±59.1	122.5±25.5
WPF	*db*/*db*	36.5±2.3	106.7±15.0	121.6±37.0**	94.8±24.9*

N=6-8, mean±S.D., *: $p<0.05$, **: $p<0.01$.

[Method of Experiment]

Mice (C57BL/KsJ-db/db, male, 9-week old) were fed walnut polyphenol-rich fraction 200mg/kg/day for 4-week. After 4-week, mice were placed in individual metabolic cages for urine collection for 8 hours and blood sample was collected after overnight starvation. Urine 8-OHdG, blood glucose and triglyceride levels were measured respectively.

Fukuda T., *et al.*, Effect of the walnut polyphenol fraction on oxidative stress in type 2 diabetes mice. *BioFactors* **21**, 251-253 (2004).

5. Other Functional Activities

(I) Skin-lightening activity (Inhibition of hyperpigmentation)

The effect of Walnut Polyphenols on skin hyperpigmentation was examined using B16 melanoma cells *in vitro*. Pre-cultured B16 melanoma cells were incubated in medium containing Walnut Polyphenols and melanin cells formation was determined. As illustrated below, Walnut Polyphenols inhibited melanin formation at concentration 1 to 30μg/mL. Apparently, Walnut Polyphenols is more superior than the popular skin-lightening agent, ascorbic acid and arbutin upon data comparison (Fig. 15)

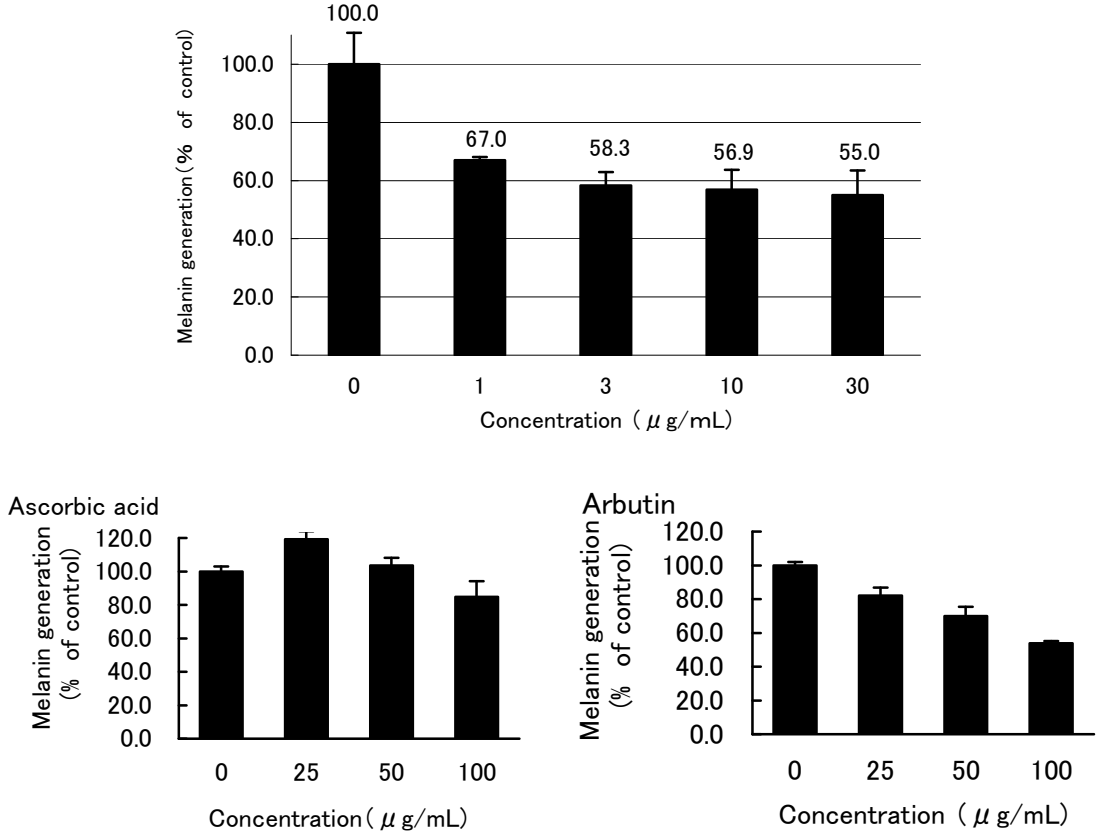


Fig. 15. The Effect of Walnut Polyphenols, Ascorbic acid and Arbutin on B16 Melanoma cells. (mean±S.D., n=5)

Data of The Effect of Ascorbic Acid & Arbutin on B16 Melanoma cells were cited from: Aitani M. and Shimoda H. *Japan Food Science*, **44**, 58-63 (2005).

(II) Suppression of bone absorption

Alpha-linolenic acid (omega 3 fatty acid) which is rich in walnut was reported to improve bone condition. To evaluate plant-derived omega 3 fatty acid rich diet on bone metabolism in human, serum N-teropeptide (NTx) and bone specific alkaline phophatase (BSAP) were determined as bone absorption and remodeling markers, respectively. Six-weeks ingestion of the diet significantly reduced NTx (Fig. 16). The result indicates that ingestion of omega 3 fatty acid rich diet suppress bone absorption.

Griel A. E., Kris-Etherton P. M., Hilpert K. F., Zhao G., West S. G., Corwin R. L. An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans. *Nutr. J.* 6:2 (2007).

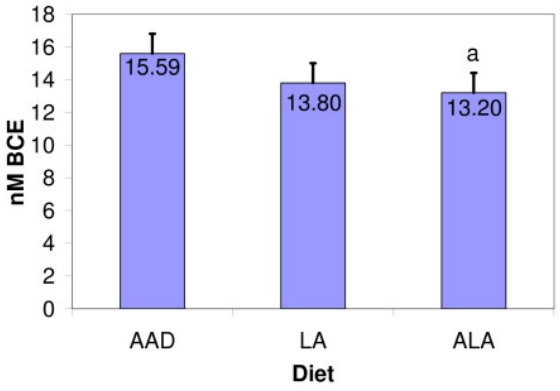


Fig. 16. The serum NTx after ingestion of the diets (mean±S.E., n=23)
AAD: Common American diet, LA: linolic acid rich diet, ALA: alpha-linolenic acid rich diet, a: significant difference: $p<0.05$.

6. Stability of Walnut Polyphenols

(I) Thermostability

Fig. 17 illustrated data of Thermostability of Walnut Polyphenols (without binder). The polyphenols content remained stable at temperature 100°C and 120°C for 1 hour. Walnut Polyphenols is highly stable.

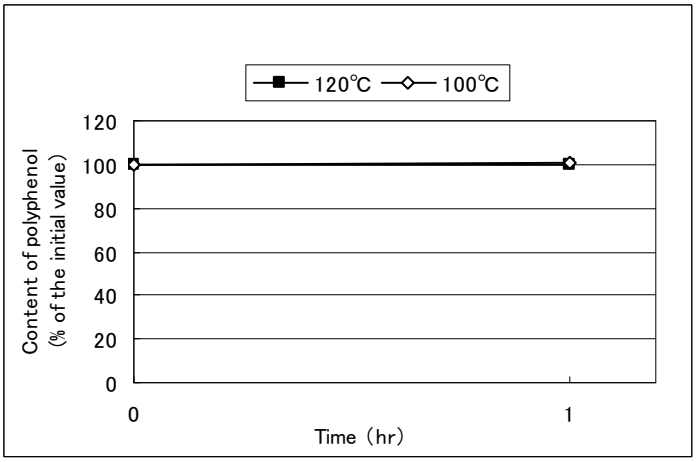


Fig. 17. Thermostability of Walnut Polyphenols

(II) pH Stability

Fig. 18 illustrated data of pH Stability of Walnut Polyphenols on Day 1 and on Day 7. Walnut Polyphenols solution (0.5%) was prepared and stored under different pH at room temperature for 1-day and 1-week. Results showed that Walnut Polyphenols is highly stable at acidic and neutral conditions but degraded at alkaline condition.

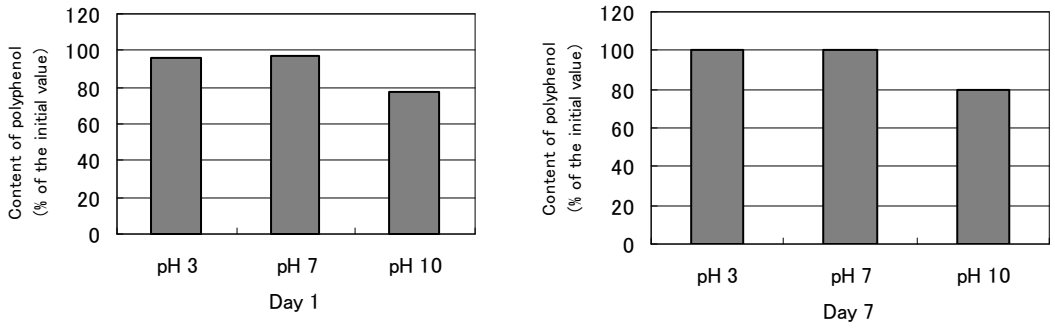


Fig. 18. pH stability of Walnut Polyphenols

7. Nutrition Information (Walnut Polyphenols)

	-P10, -WSP10	Note	Method
Water	1.9 g/100g		Vacuum superheating drying method
Protein	1.5 g/100g	1	Kjeldahl method
Fat	14.0 g/100g		Acid decomposition
Ash	1.7 g/100g		Direct incineration
Carbohydrate	82.5 g/100g	2	
Energy	464 kcal/100g	3	Modified Atwater method
Dietary fiber	1.2> g/100g		Prosky method
Sodium	203 mg/100g		Atomic absorption spectrophotometory

1） Nitrogen protein conversion factor: 6.25

2） Calculation：100 – (water + protein + fat + ash)

3） Energy expression standard：protein 4; fat 9; sugar 4; dietary fat2

Test trustee：SRL

Data：September 15, 2006

Report No.：2006090400032

8. Safety Profile of Walnut Polyphenols

(I) Residual Agricultural Chemical

Walnut Polyphenols (without binder) is conformed to regulation stipulated for 447 residual agricultural chemical compounds. No residual agricultural chemicals detected as confirm by test trustee.

Test trustee：Masis Co. Ltd.

Data：September 4, 2006

Report No.：7035

(II) Acute Toxicity（LD_{50}）

Fasting male and female mice (ddY, 5-week old) were given orally 2,000mg/kg Walnut Polyphenols (no binder) in accordance to Single Dose Toxicity Test Guideline for Pharmaceuticals. Mice were maintained for observation for 14 days. No fatal event occur nor abnormal changes observed upon comparison with control group. No evident abnormalities detected in organs upon autopsy. Oral LD_{50} of Walnut Polyphenols is deduced to be >2,000mg/kg for both male and female mice.

(III) Human Consumption Test

4 male volunteers were given oral Walnut Polyphenols (without binder) 50mg/day for 4 weeks. Blood profile screening was carried out for analysis prior to and after the test. No abnormal reading detected in blood profile screening.

Blood profile screening: Total bilirubin, Total protein, Albumin, AST, ALT, LDH, LAP, γ-GTP, cholinesterase, amylase, lipase, L-CAT, LDL-cholesterol, total cholesterol, triglyceride, phospholipid, FFA, HDL-cholesterol, Na, K, serum Fe, TIBC, UIBC, urea nitrogen, uric acid, glucose, hemocytes.

9. Dosage Recommendation

The recommended daily dosage of Walnut Polyphenol-P10 & Walnut Polyphenol-WSP10 is 50 to 150 mg

10. Crude Material Equivalent

1g of Walnut Polyphenols is equivalent to 200 edible portion of walnuts. Recommended daily consumption of walnut is 10 – 30 walnuts.

11. Commercial Application

	Application	Claim	Example
Foods	Hepatoprotection, prevention of metabolic syndrome, anti-oxidation, beautifying	Hepatoprotection, prevention of metabolic syndrome, diabetes, hyperlipidemia, hypertension, anti-oxidation, whitening	Beverages, hard & soft capsules, tablets, candies, chewing gum, chocolates, wafers, jellies, etc
Cosmetics	Whitening		Body lotion, body gel, etc

12. Packaging

WALNUT POLYPHENOL-P10（powder, food grade）, -WSP10（water-soluble powder, food grade）

5kg Interior package：aluminium bag
Exterior package：cardboard box

WALNUT POLYPHENOL-PC10（powder, cosmetics grade）, -WSPC10（water-soluble powder, cosmetics grade）

5kg Interior package：aluminium bag
Exterior package：cardboard box

WALNUT POLYPHENOL-LC（water-soluble liquid, cosmetics grade）

5kg Interior package：cubic polyethylene container
Exterior package：cardboard box

WALNUT SEED OIL（oil and food）

16kg Interior package：tinplate can
Exterior package：cardboard box

13. Storage

Store in cool, dry dark place

14. Expression

<Food>

WALNUT POLYPHENOL -P10, -WSP10

Example：walnut extract, walnut polyphenol

WALNUT SEED OIL

Example：walnut oil, walnut seed oil

<Cosmetics>

WALNUT POLYPHENOL -PC10, -WSPC10

INCI name：Dextrin, Juglans Regia (Walnut) Seedcoat Extract

WALNUT POLYPHENOL -LC

INCI name：Butylene Glycol, Water, Juglans Regia (Walnut) Seedcoat Extract

PRODUCT STANDARD
PRODUCT NAME

WALNUT POLYPHENOL-P10

Food

This product is extracted with aqueous ethanol from seed coats of walnut (*Juglans regia* L.) or its congeners. It guarantees minimum 10.0 % polyphenols and 0.1% tellimagrandin I.

Appearance	Light brown to dark brown powder with slightly unique smell	
Polyphenols	Min. 10 %	(Folin-Denis method)
Tellimagrandin I	Min. 0.1 %	(HPLC)
Loss on drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105℃, 2 hr)
Purity test		
(1) Heavy metals (as Pb_2)	Max. 20 ppm	(Sodium Sulfide Colorimetric Method)
(2) Arsenic (as As_2O_3)	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation, The Third Method, Apparatus B)
Standard Plate Counts	Max. 3×10^3 cfu / g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^3 cfu/ g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Dextrin	67 %
Walnut extract	33%
Total	100 %

PRODUCT STANDARD
PRODUCT NAME

WALNUT POLYPHENOL-WSP10

Food

This water-soluble product is extracted with aqueous ethanol from seed coats of walnut (*Juglans regia* L.) or its congeners. It guarantees minimum 10.0 % polyphenols.

Appearance	Light brown to dark brown powder with slightly unique smell	
Polyphenols	Min. 10 %	(Folin-Denis method)
Tellimagrandin I	Identification of peak	(HPLC)
Loss on drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105℃, 2 hr)
Purity test		
(1) Heavy metals (as Pb_2)	Max. 20 ppm	(Sodium Sulfide Colorimetric Method)
(2) Arsenic (as As_2O_3)	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation, The Third Method, Apparatus B)
Standard Plate Counts	Max. 3×10^3 cfu / g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^3 cfu/ g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Maltodextrin	67 %
Walnut extract	33 %
Total	100 %

PRODUCT STANDARD
PRODUCT NAME

WALNUT POLYPHENOL-PC10

Cosmetics

This product is extracted with aqueous ethanol from seed coats of walnut (*Juglans regia* L.) or its congeners. It guarantees minimum 10.0 % polyphenols and 0.1% tellimagrandin I.

Appearance	Light brown to dark brown powder with slightly unique smell	
Polyphenols	Min. 10 %	(Folin-Denis method)
Tellimagrandin I	Min. 0.1 %	(HPLC)
Loss on drying	Max. 10.0 %	(1 g, 105℃, 2 hr)
Purity test		
(1) Heavy metals (as Pb_2)	Max. 10 ppm	(The second method of The Japanese Standards of Quasi-Drug Ingredients)
(2) Arsenic (as As_2O_3)	Max. 1 ppm	(The third method of The Japanese Standards of Quasi-Drug Ingredients)
Standard Plate Counts	Max. 1×10^2 cfu / g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/ g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Dextrin	67 %
Juglans Regia (Walnut) seed coat extract	33 %
Total	100 %

PRODUCT STANDARD
PRODUCT NAME

WALNUT POLYPHENOL-WSPC10

Cosmetics

This water-soluble product is extracted with aqueous ethanol from seed coats of walnut (*Juglans regia* L.) or its congeners. It guarantees minimum 10.0 % polyphenols.

Appearance	Light brown to dark brown powder with slightly unique smell	
Polyphenols	Min. 10 %	(Folin-Denis method)
Tellimagrandin I	dentification of peak	(HPLC)
Loss on drying	Max. 10.0 %	(1 g, 105℃, 2 hr)
Purity test		
(1) Heavy metals (as Pb_2)	Max. 10 ppm	(The second method of The Japanese Standards of Quasi-Drug Ingredients)
(2) Arsenic (as As_2O_3)	Max. 1 ppm	(The third method of The Japanese Standards of Quasi-Drug Ingredients)
Standard Plate Counts	Max. 1×10^2 cfu / g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/ g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Dextrin	67 %
Juglans Regia (Walnut) seed coat extract	33 %
Total	100 %

PRODUCT STANDARD
PRODUCT NAME

WALNUT POLYPHENOL-LC

Cosmetics

This product is extracted after defatting the seed coat of walnut (Juglans regia L.) with hexane, adding anethanol solution completely removing ethanol, add 1,3-butylene glycol water solution into filtrate as the product.

Appearance	Brown liquid with slightly unique smell	
Certification test (Polyphenols)	Dissolve 30 μL of this product in 3.5 mL water. Add 0.2 mL Folin-Denis reagent into the solution followed by 0.4 mL saturated Na_2CO_3. The solution changes to blue.	
Purity test		
(1) Heavy metals (as Pb_2)	Max. 10 ppm	(The second method of The Japanese Standards of Quasi-Drug Ingredients)
(2) Arsenic (as As_2O_3)	Max. 1 ppm	(The third method of The Japanese Standards of Quasi-Drug Ingredients)
Standard Plate Counts	Max. 1×10^2 cfu / g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/ g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Water	50 %
Butylene Glycol	49 %
Juglans Regia (Walnut) seed coat extract	1 %
Total	100 %

PRODUCT STANDARD
PRODUCT NAME

WALNUT SEED OIL

Food

This product is oil extracted from seeds of walnuts, the seeds of *Juglans regia* Linne and subsequently refined.

Appearance	Clear oil of light yellowish color with slightly unique smell。	
α-linoleic acid	Min. 10%	
Acid value	Max. 0.5	
Iodine value	140 to 183	
Saponification value	188 to 196	
Color	Max. 5.0 - 50	(Gardoner method)
Purity test		
(1) Heavy metals (as Pb_2)	Max. 10 ppm	(Sodium Sulfide Colorimetric Method)
(2) Arsenic (as As_2O_3)	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation, The third method, Apparatus B)
Standard Plate Counts	Max. 1×10^2 cfu / g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Negative	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)
Composition	Walnut seed oil 100 %	

ORYZA OIL & FAT CHEMICAL CO., LTD. striving for the development of the new functional food materials to promote health and general well-being.

From product planning to OEM - For any additional information or assistance, please contact：

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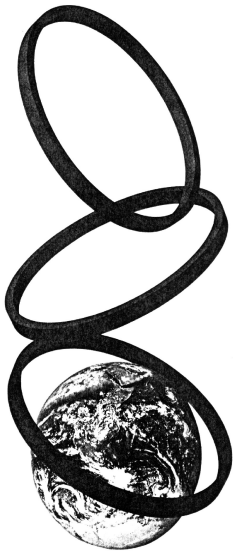
Established Date: January 12, 2007
Revised Date: December 27,2019



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