Oryza

ORYZA OIL & FAT CHEMICAL CO., LTD.

WATER SHIELD EXTRACT

Prevention of formation of cellulite
Improvement of metabolism
Prevention of metabolic syndrome

- WATER SHIELD EXTRACT-P
  (Powder for foods)
- WATER SHIELD EXTRACT -WSP
  (Water-soluble powder for foods)
- WATER SHIELD EXTRACT -PC
  (Powder for cosmetics)
- WATER SHIELD EXTRACT -WSPC
  (Water-soluble powder for cosmetics)
- WATER SHIELD EXTRACT -LC
  (Water-soluble liquid for cosmetics)

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Ver. 1.1 YF

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WATER SHIELD EXTRACT

1. Introduction

Water shield or scientifically known as *Brasenia schreberi*, is an perennial aquatic plant floating, peltate leaves and rhizomatous stems (Fig. 1 left). Mie-cho of Akita Pref is the major production site of Water Shield plant and it is also an important edible food material for fine cuisine. Traditionally, the harvesting period of Water Shield buds started in early summer using large basin collecting from swampy water shield plantation (Fig. 1 right). Water Shield plant is covered with mucilaginous gel which is rich in polysaccharides[1)] and gives good texture. The low caloric count of Water Shield has projected it as a healthy and luxurious food ingredient. In recent years, collaborative research between Harvestech Corporation and Akita Pref. Food Research Centre reported the cholesterol and triglyceride lowering effect of Water Shield. There is increasing researches prompted to study the effect of Brasenia on metabolic syndrome in accordance to the polyphenol content of the plant.

Research conducted at Oryza Oil & Fat Chemical Co., Ltd. revealed that Water Shield Extract demonstrated excellent inhibitory effect on fat accumulation as well as cell damage due to excessive fat accumulation. Water Shield Extract contains variety of functional compounds such as flavonoids and polyphenols. Scientifically, it prevents fat accumulation and suppresses the unsightly formation of cellulite on skin. In collaboration with Harvestech Corporation and Akita Pref. Food Research Centre, Oryza Oil & Fat Chemical Co., Ltd. successfully developed, produced and commercialized Water Shield Extract, a newly discovered health ingredients for the prevention of metabolic syndrome and associated disorders.

Fig. 1. Water shield (left) and the harvesting (right)

1) Kakuta, M.; Misaki A. Structual characterization of water-shield mucilage of “Junsai” (*Brasenia schreberi* J. F. Gmel). *Foods Food Ingredients J. Jpn.* **2004**, *209*, 298−303.
2) Takahashi, J.; Toshima, G.; Matsumoto, Y.; Kimura, F.; Kiuchi, T.; Hamada, K.; Hata, K. *In vitro* screening for antihyperlipidemic activities in foodstuffs by evaluating lipoprotein profiles secreted from human hepatoma cells. *J. Nat. Med*., **2011**, *65*, 670−674.

2. Functional Components of Water Shield Extract (*Brasenia schreberi*) and newly discovered compound – Junsainoside A

The gel-like component covering the surface of Water Shield plant is polysaccharide, while the leaves of Water Shield is rich in polyphenols (>30% in pure extract without excipients). Previously, in a study conducted on Water Shield produced in Canada, gallic acid, quercetin and quercetin 7-*O*-glucoside was isolated [3]. However, detailed study on the components of Water Shield was unknown. In collaboration with Kyoto Pharmaceutical University, we ventured into the research and analysis of Water Shield produced by Mie-cho, Akita Pref. Fig. 2 showed the chemical structures of isolated compounds of *Brasenia schreberi*. Furthermore, a new compound was discovered from the research work and we named the compound as junsainoside A.

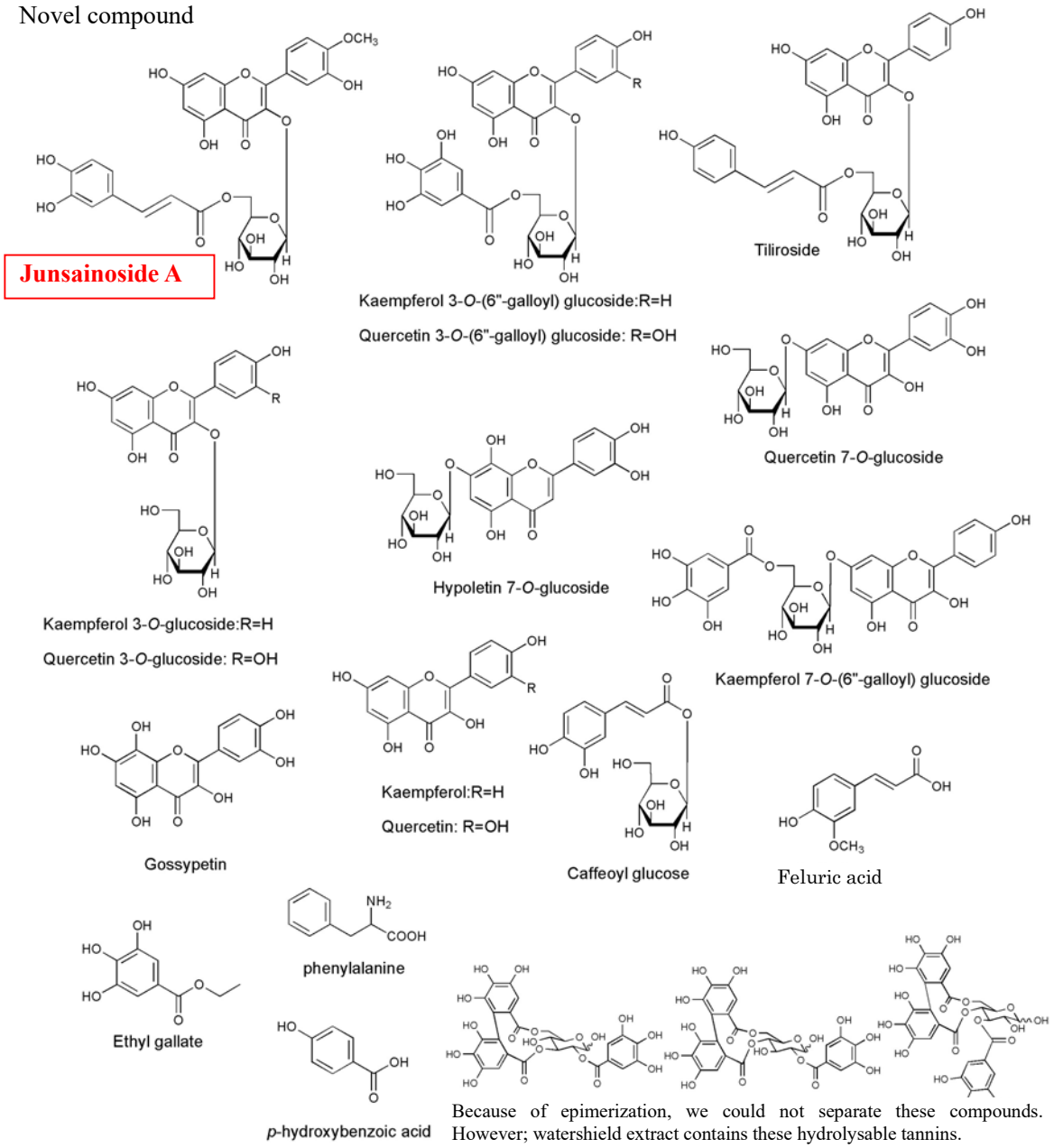
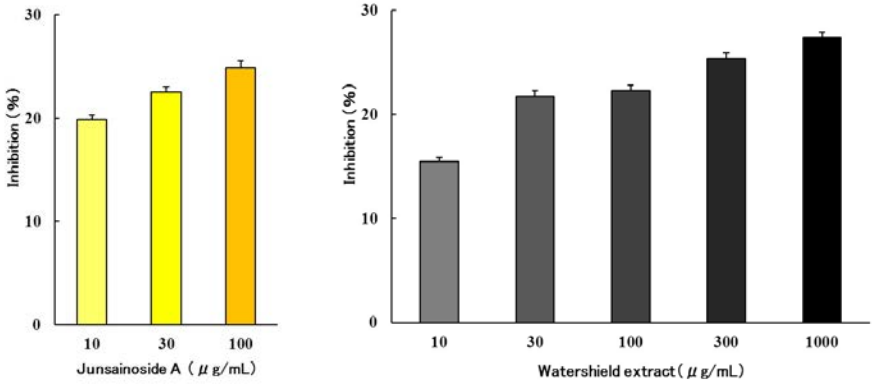


Fig. 2. Compounds in water shield

3) Legault J., Perron T., Mshvildadze V., Girard-Lalancette K., Perron S., Laprise C., Sirois P., Pichette A. Antioxidant and anti-inflammatory activities of quercetin 7-*O*-β-D-glucopyranoside from the leaves of *Brasenia schreberi*. *J. Med. Food*, 14, 1127-34 (2011).

Further experiment was prompted to investigate the functional effect of Junsainoside A, results showed that Junsainoside A significantly inhibited the activity of collagenase and elastase, preventing the degradation of collagen and elastin respectively (Fig. 3). Based on above findings, it is suggestive that Junsainoside A prevents wrinkle formation and anti-ageing. As illustrated in Fig. 4, the HPLC spectra of Water Shield Extract showed that Junsainoside A exists as the major component, hence Water Shield Extract is commercialized with standardized Junsainoside A as major functional component.

A) Inhibition of collagenase



B) Inhibition of elastase

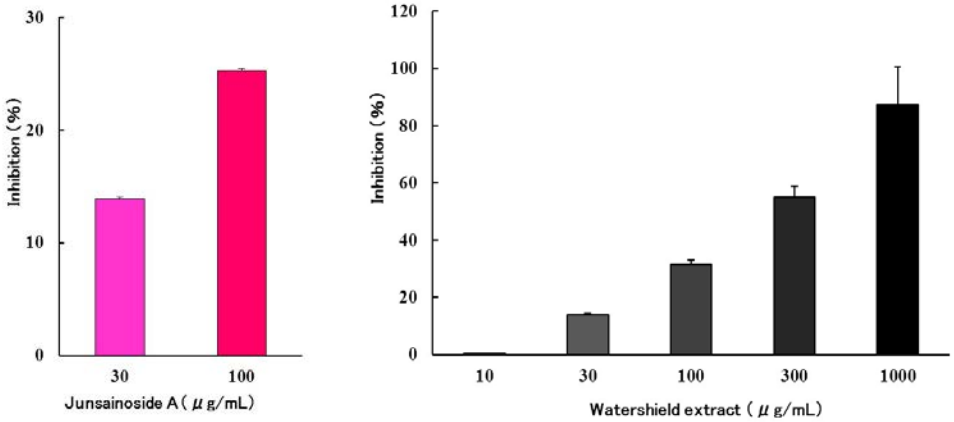


Fig. 3. Inhibitory effect of watershield extract on collagenase and elastase activities
Mean±SE

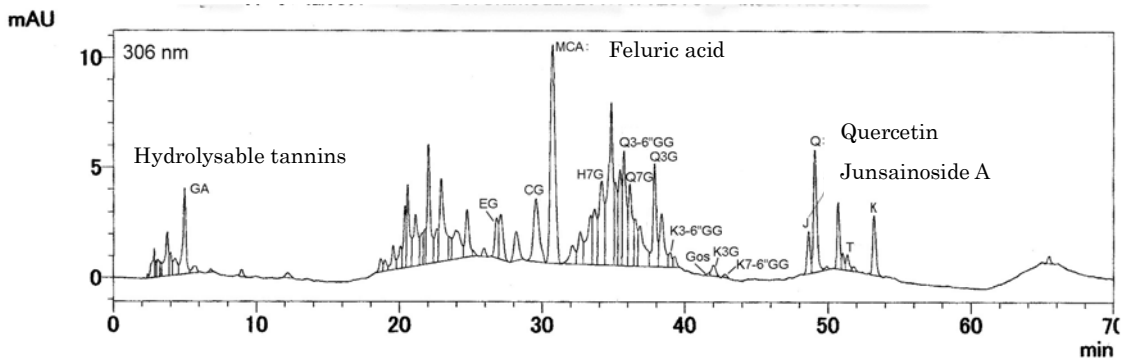


Fig. 4. HPLC chromatogram of water shield extract

Solvents : Stepwise gradient of 15%MeOH containing 1 mM phosphoric acid and15%MeOH containing 1 mM phosphoric acid, column : ODS (4.6 × 250 mm), flowlate : 1 mL/min, wavelength : 306 nm, GA: gallic acid, EG: ethyl gallate, CG: caffeoyl glucose, MCA: ferulic acid, H7G: hypoletin 7-*O*-glucoside, Q3-6"GG: quercetin 3-*O*-(6"-galloyl) glucoside, Q7G: quercetin 7-*O*-glucoside, Q3G: quercetin 3-*O*-glucoside, K3-6"GG: kaempferol 3-*O*-(6"-galloyl) glucoside, Gos: gossypetin, K3G: kaempferol 3-*O*-glucoside, K7-6"GG: kaempferol 7-*O*-(6"-galloyl) glucoside, J: junsainoside A, Q: quercetin, T: tiliroside, K: kaempferol

As mentioned, Water Shield Extract is rich in polyphenols with potent antioxidative activity, Table 1. showed the effect of Water Shield Extract and its polyphenols in response to reactive oxygen species (ROS) production in NB1RGB fibroblasts cells. ROS production is responsible in driving the ageing process causing reduced level of collagen and elastin, affecting type I and II collagen in the dermis and breakdown of extracellular matrix (ECM) proteins [4]. Water Shield Extract and its polyphenols demonstrated inhibitory effect on oxidation of ECM proteins, thus is suggestive that to slow down the process of ageing.

4) Callaghan T.M., Wilhelm K.P. A review of ageing and an examination of clinical methods in the assessment of ageing skin. Part I: Cellular and molecular perspectives of skin ageing. *Int. J. Cosmet. Sci.* 30, 313-22 (2008).

Table 1. The effect of Water Shield Extract and its polyphenols on ROS production in NB1RGB fibroblasts cells.

	Conc. (μg/mL)	ROS production (% of control)
Normal	-	116.0±3.4**
Control	-	100.0±2.7
Water shield extract	1	89.0±1.9**
	10	69.5±0.9**
	(μM)	
Normal	-	112.9±3.2**
Control	-	100.0±2.8
Ethyl gallate	1	86.6±2.1**
	10	67.8±0.5**
Caffeoyl glucose	1	83.1±2.2**
	10	53.2±0.3**
Ferluic acid	1	88.2±3.4
	10	66.4±2.4**
Hypolaetin 7-*O*-glucoside	1	81.1±2.8**
	10	52.2±1.0**
Quercetin 3-*O*-(6"-galloyl)-glucoside	1	67.6±1.8**
	10	44.7±0.5**
Quercetin 3-*O*-glucoside	1	86.7±2.4**
	10	59.9±1.6**
Kaempferol 3-*O*-(6"-galloyl)-glucoside	1	86.2±3.0**
	10	55.9±1.9**
Gossypetin	1	82.8±3.5**
	10	54.8±0.9**
Kaempherol 3-*O*-glucoside	1	90.1±4.6
	10	91.9±3.1
Junsainoside A	1	95.5±1.6
	10	84.4±1.1**
Quercetin	1	94.8±2.2
	10	87.5±1.0**
Tiliroside	1	98.2±2.1
	10	101.1±2.4
Kaempferol	1	94.1±1.2*
	10	62.4±0.5**

Mean±SE, n=6, *: $p<0.05$, **: $p<0.01$

3. The relationship between skin cells and subcutaneous fat

Recently, the study on the relationship between skin cells and subcutaneous fat as captured tremendous interest. [5] Of course, increase fat intake will inevitably increase the amount of subcutaneous fat which ultimately leads to the formation of cellulite and sagging skin. In addition, collagen and elastin production is inhibited when fibroblasts cells surrounding subcutaneous fat is damaged by TNF-α production. [6] On the other hand, studies showed that TNF-α enhances the degradation of collagen by collagenase. [7] Meanwhile, adiponectin which is exclusively secreted from adipose tissue has been reported to promote hyaluronan synthesis [8,9] and extracellular matrix formation. [10] Decrease of adiponectin will thus inhibit the production if hyaluronic acid. Nonetheless, production of indole, [10,11] ammonia [12] and other phenols increases with excessive fat intake causing disturbances of the intestinal flora which exacerbate skin condition with abnormal keratinization of epidermal cells. Fig. 5 illustrated the mechanism of action ① - ⑤ of Water Shield Extract in preventing damage of skin cells surrounding subcutaneous fat in response to excessive fat intake.

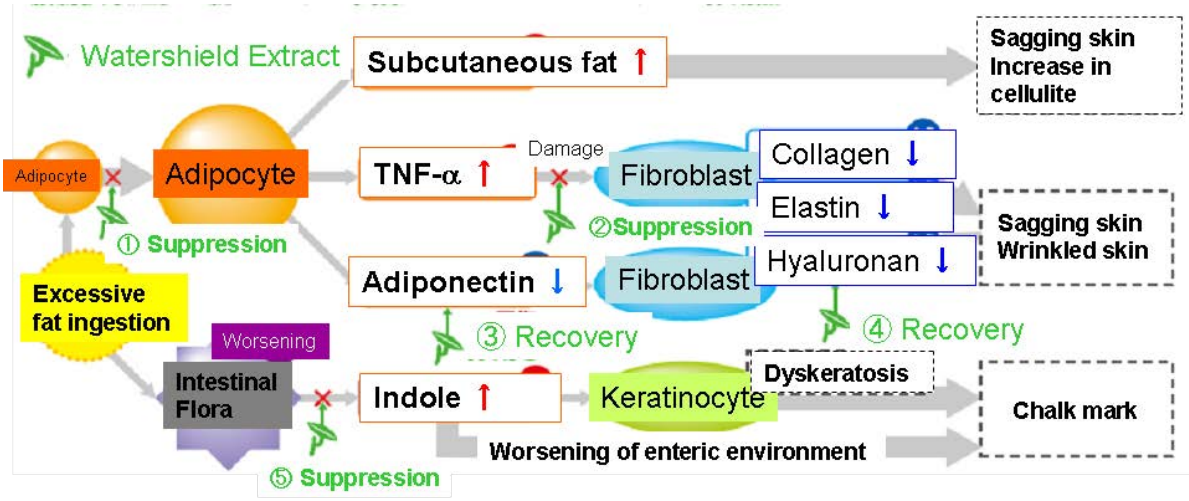


Fig. 5. The mechanism of action of Water Shield Extract on skin in response to excessive fat intake.

5) Minamino M., Iizuka R., Chiba M Intestinal flora and skin physiology. *J. Jpn. Cosmetic Sci. Soc.*, 35, 325-31 (2011).
6) Zhu G., Cai J., Zhang J., Zhao Y., Xu B. Abnormal nuclear factor (NF)-κB signal pathway and aspirin inhibits tumor necrosis factor alpha-induced NF-κB activation in keloid fibroblasts. *Dermatol Surg.*, 33, 697-708 (2007).
7) Lindner D., ZietschC., Becher P.M., Schulze K., Schultheiss H-P., Tschöpe C., Westermann D. Differential expression of matrix metalloproteases in human fibroblasts with different origins. *Biochem. Res. Int.*, Article ID 875742 (2012).
8) Akazawa Y., Sayo Y., Sugiyama Y., Sato T., Akimoto N., Ito A., Inoue S. Adiponectin resides in mouse skin and up-regulates hyaluronan synthesis in dermal fibroblasts. *Connective Tissue Res.*, 52, 322-8 (2011).
9) Yamane T., Kobayashi-Hattori K., Oishi Y. Adiponectin promotes hyaluronan synthesis along with increase in hyaluronan synthase 2 transcripts through an AMP-activated protein kinase/peroxisome proliferator-activated receptor-α-dependent pathway in human dermal

fibroblasts. *Biochem. Biophys. Res. Commun.*, 415, 235-8 (2011).
10) Ezure T., Amano S. Adipnectin and leptin up-regulate extracellular matrix production by dermal fibroblasts. BioFactors, 31, 229-36 (2007).
11) Kakino M., Sugiyama T., Kunieda H., Tazawa S., Maruyama H., Tsuruma K., Araki Y., Shimazawa M., Ichihara K., Mori H., Hara H. Agarwood (*Aquilaria crassna*) extracts decrease high-protein high-fat diet-induced intestinal putrefaction toxins in mice. *Pharm. Anal. Acta*., 3: 152, 2153-435 (2012).
12) Iizuka R., Kawakami K., Izawa N., Chiba K. Phenols produced by gut bacteria affect the skin in hairless mice. *Microbial Ecolo. Health Disease*, 21, 50-6 (2009).

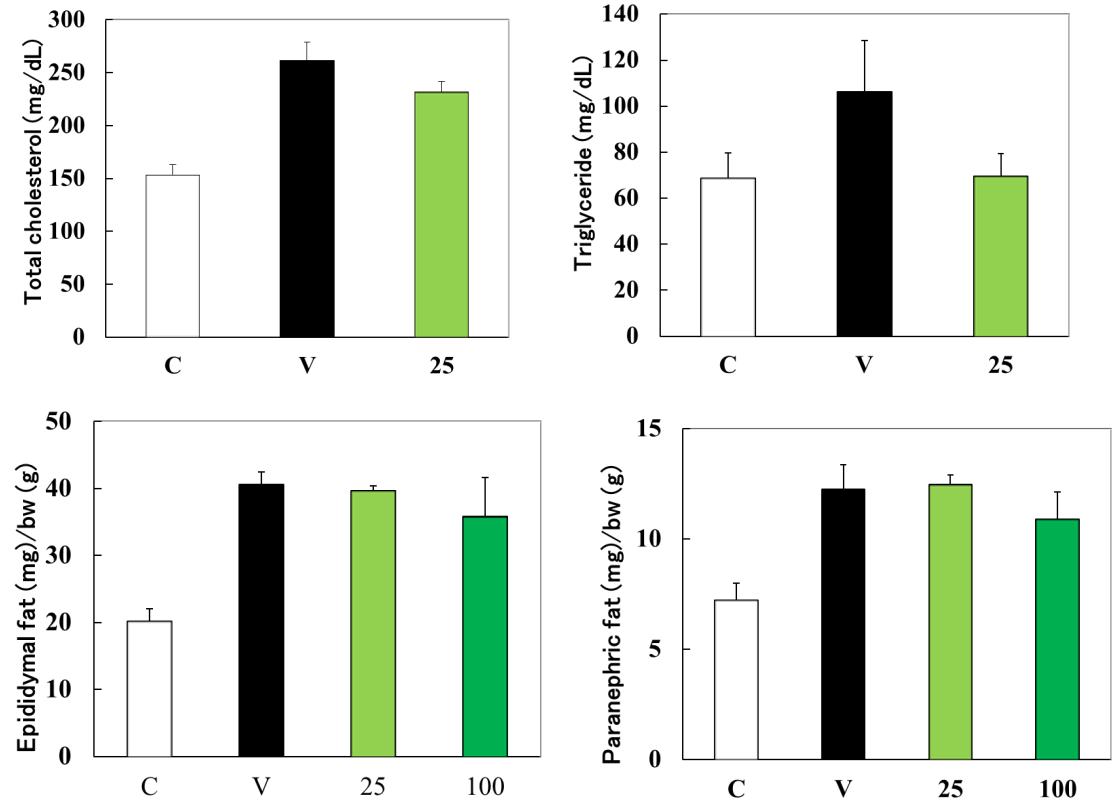
Following index is the page of function of Water Shield Extract

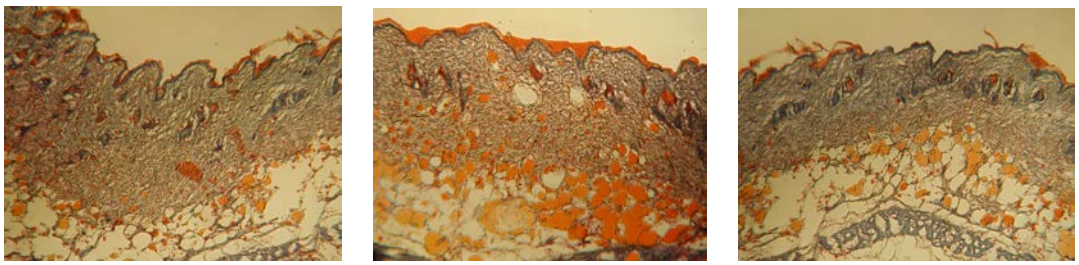
4. The Effect of Water Shield Extract on fat cells and skin cells with excessive fat intake

i Prevention of Fat Accumulation

a. Effect on mice fed with high lipid diet

Mice fed with high lipid diet (HFD) were treated orally with Water Shield Extract (without excipients) at 25mg/kg/day and 100mg/kg/day for 14 days. Blood cholesterol was analyzed, weight of visceral fat and subcutaneous fat was measured at the end of experiment. Result showed that blood cholesterol and triglyceride reduced in mice treated with 25mg/kg/day of Water Shield Extract. Meanwhile, epididymal fat and paranephric fat reduced in mice treated with 100mg/kg/day of Water Shield Extract (Fig. 6). Cross-section images of stained cells clearly showed a reduced fat area (RED dye).





C （normal diet） V （HFD） Water Shield Extract 25 mg/kg

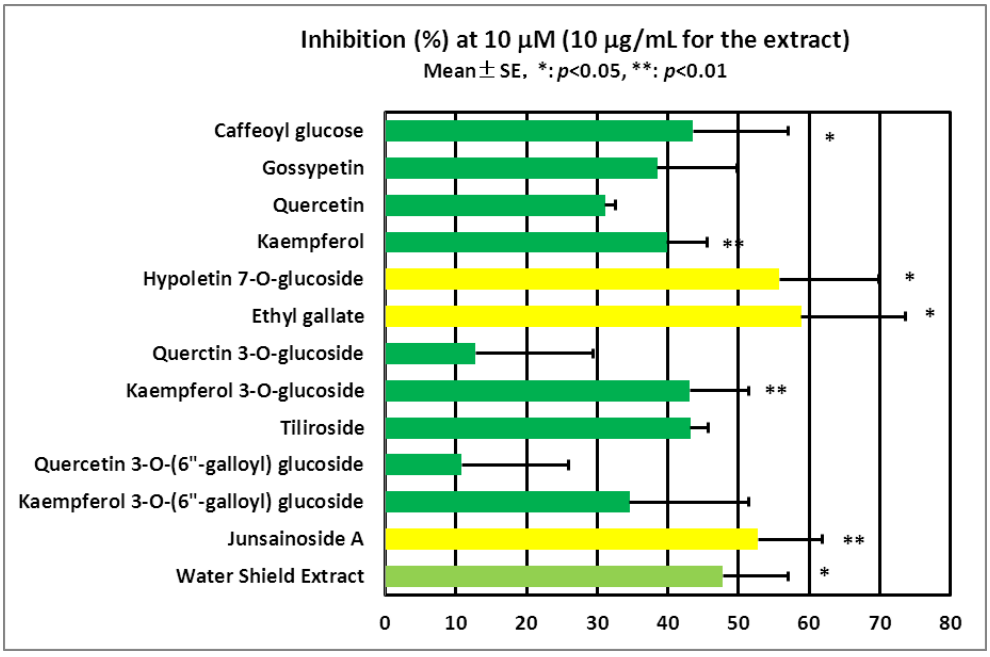
Fat in dermis was decreased by Water Shield Extract

Fig. 6 The effect of Water Shield Extract on blood cholesterol, visceral fat and subcutaneous fat of mice fed with high lipid diet (HFD). Mean with the SE (n=5-7)

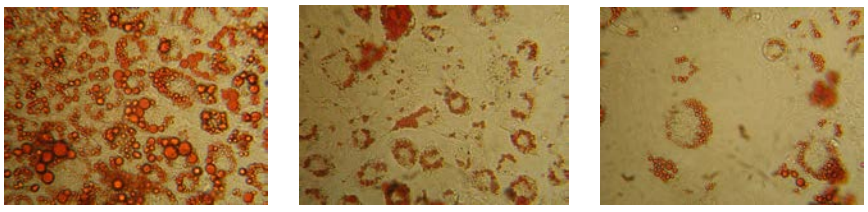
b. Prevention of visceral fat production and fat accumulation of cultured cells

3T3-L1 cells (adipose cells which increase the synthesis and accumulation of triglyceride) was cultured in medium containing Water Shield Extract. The amount of fat accumulated was determined at the end of the experiment. As illustrated in Fig. 7 A & B, Water Shield Extract at 10μg/mL inhibited 50% of fat accumulation in 3T3-L1 cells. The functional compounds, Junsainoside A, ethyl gallate and hypoletin 7-*O*-glucoside similarly inhibited 50% of fat accumulation. Other compounds such as kaempferol 3-*O* glucoside, kaempferol and caffeoyl glucose showed significant inhibitory effect on fat accumulation even at low concentration of 1μM. Based on above findings, Water Shield Extract and its functional compounds is preventive of fat accumulation.

A)

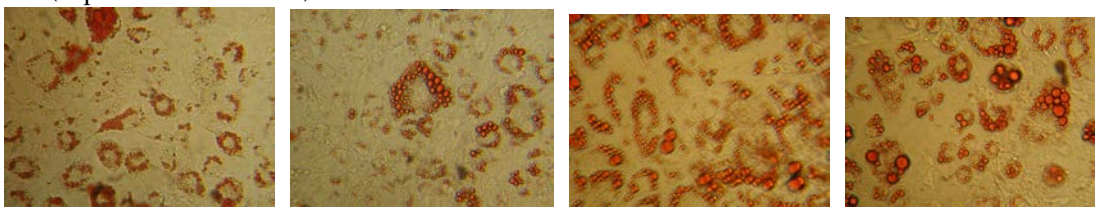


B)



Control　　1 μg/mL　　10 μg/mL

(Lipid is stained in red)　　Water Shield Extract



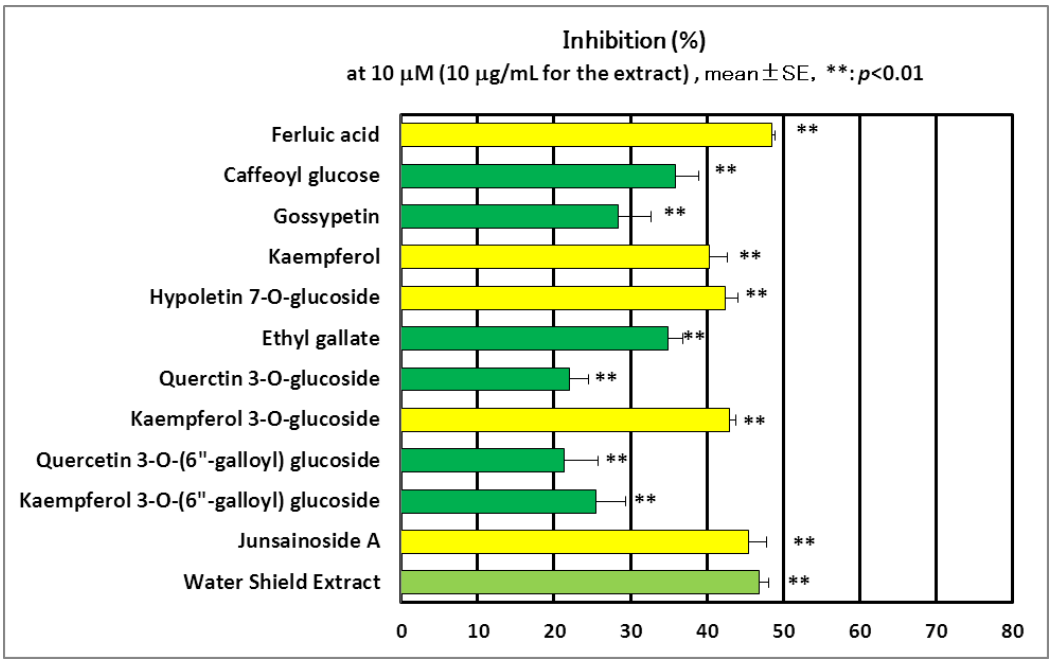
Junsainoside A　　Ethyl gallate　　Hypolaetin 7-*O*-glucoside　　Kaempferol 3-*O*-glucoside

Fig. 7　The effect of Water Shield Extract and its functional compounds on 3T3-L1 cells and fat accumulation. A: Inhibition rate of fat accumulation. B: Stained fat image (treated with 1μM concentration)

c. Prevention of subcutaneous fat accumulation of cultured cells

Adipocytes (derived from human subcutaneous adipose) were cultured in medium containing Water Shield Extract and its functional compounds. The amount of fat accumulated was determined at the end of the experiment. As illustrated in Fig. 8 A & B, Water Shield Extract at 10μg/mL inhibited 50% of fat accumulation in adipocytes. Meanwhile, its functional compounds, Junsainoside A, kaempferol 3-*O*-glucoside, hypolaetin 7-*O*-glucoside, kaempferol and methyl caffeic acid inhibited 40% of fat accumulation. Other compounds showed 20-40% inhibitory effect on fat accumulation at concentration as low as 1μM. As a result, the functional compounds of Water Shield Extract are preventive of fat accumulation (Fig. 8B).

A)



B)

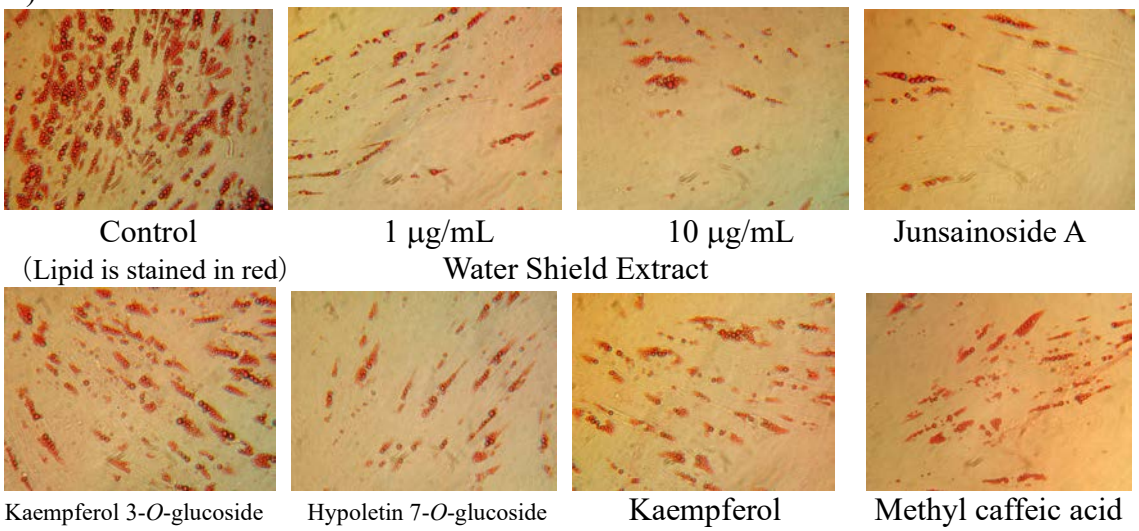


Fig. 8. The effect of Water Shield Extract and its functional compounds on human subcutaneous adipocytes and fat accumulation. A: Inhibition rate of fat accumulation. B: Stained fat image (treated with 1μM concentration)

ii. Inhibition of TNF-α induced fibroblasts damage and promotion of collagen production

Previous findings showed that Water Shield Extract and its functional compounds suppresses fat accumulation, it is suggestive that Water Shield Extract may inhibit TNF-αinduced fibroblasts damage. Further experiment is prompted to investigate the effect of Water Shield Extract on TNF-α (10nM) induced fibroblasts (NB1RGB cells) damage. Results showed that Water Shield Extract at concentration 1μg/mL effectively hindered TNF-α induced fibroblasts damage. As showed in Fig. 9, the inhibitory effect of Water Shield Extract on TNF-αinduced fibroblasts damage is dose-dependent. Meanwhile, quercetin and ethyl gallate are the functional compounds identified to inhibit fibroblasts damage.

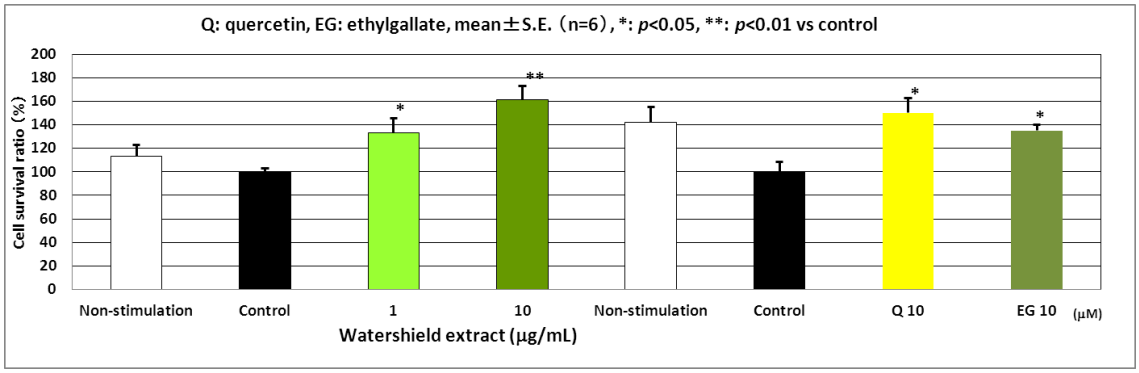


Fig. 9 The effect of Water Shield Extract and its functional compounds on TNF-α induced fibroblasts damage (Mean ± S.D.; n=4-6, *: p<0.05, **:p<0.01)

Next, the expression of type I collagen in skin of mice fed with high lipid diet (from previous experiment) was examined. As illustrated in Fig. 10, the expression of type I collagen was up-regulated in skin of mice fed with high lipid diet and Water Shield Extract. Therefore, Water Shield Extract promotes collagen production in the fibroblasts.

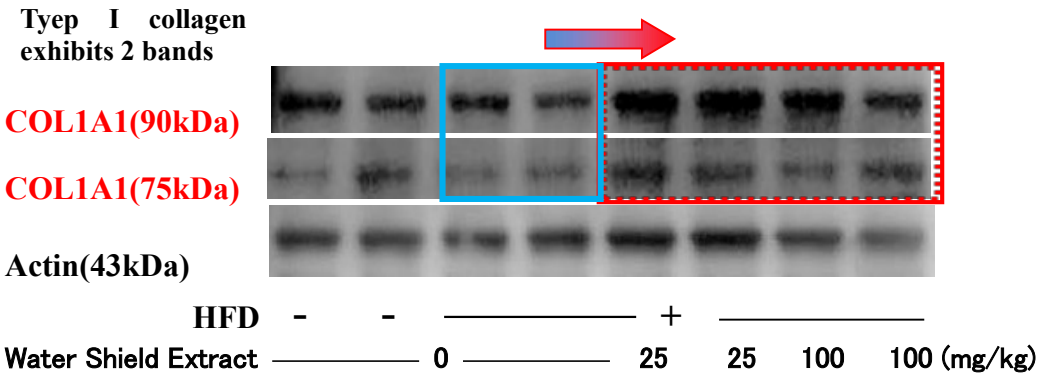


Fig. 10. Up-regulation of type I collagen by Water Shield Extract.

iii. Up-regulation on Adiponectin expression

The effect of Water Shield Extract on adiponectin production in adipocytes(fat cells) was examined. In the experiment, skin of mice fed with high lipid diet with and without Water Shield Extract was collected and the protein expression of adiponectin was evaluated. As illustrated in Fig. 11, the expression of adiponectin was up-regulated in skin of mice fed with high lipid diet and Water Shield Extract (25mg/kg).

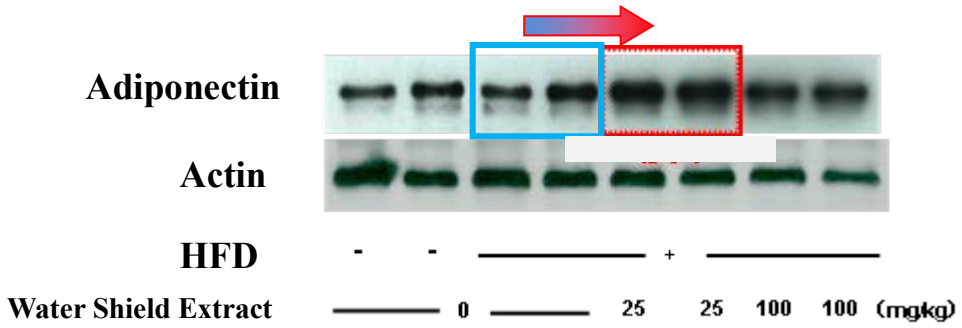
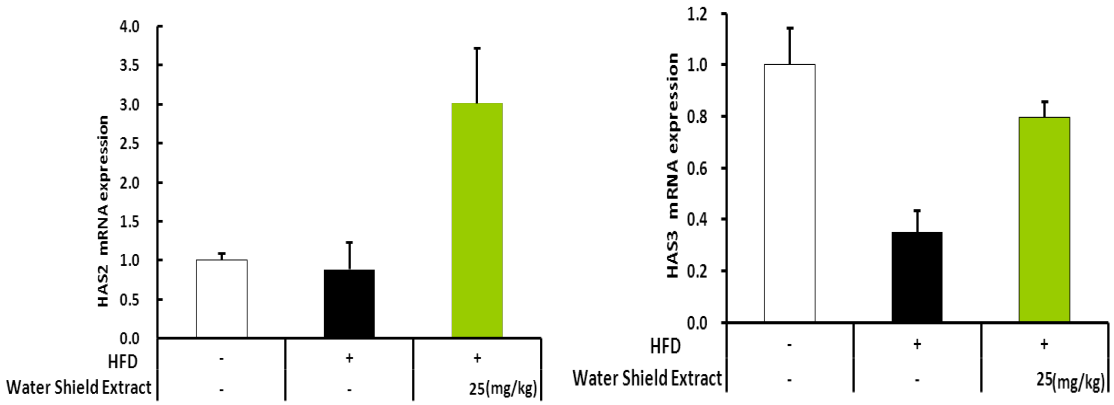


Fig. 11 The Effect of Water Shield Extract on the expression of adiponectin in mice fed with high lipid diet.

iv. Improvement on the production of Hyaluronic Acid

Moving ahead from the findings on up-regulation of adiponectin expression, the effect of Water Shield Extract on the production of hyaluronic acid was examined. Hyaluronan synthase 2 & 3(HAS) is the enzyme encoded by the by HAS2 and HAS 3 respectively for hyaluronan synthesis. Similarly, skin of mice fed with high lipid diet with and without Water Shield Extract was collected for genetic expression analysis. Results showed that the expression of HAS2 (mainly expressed in the dermis of skin) and HAS3 (mainly expressed in the epidermis) has been up-regulated respectively in mice fed with high lipid diet and Water Shield Extract. It is suggestive that Water Shield Extract improved and promoted the synthesis of hyaluronic acid in the skin.

A)



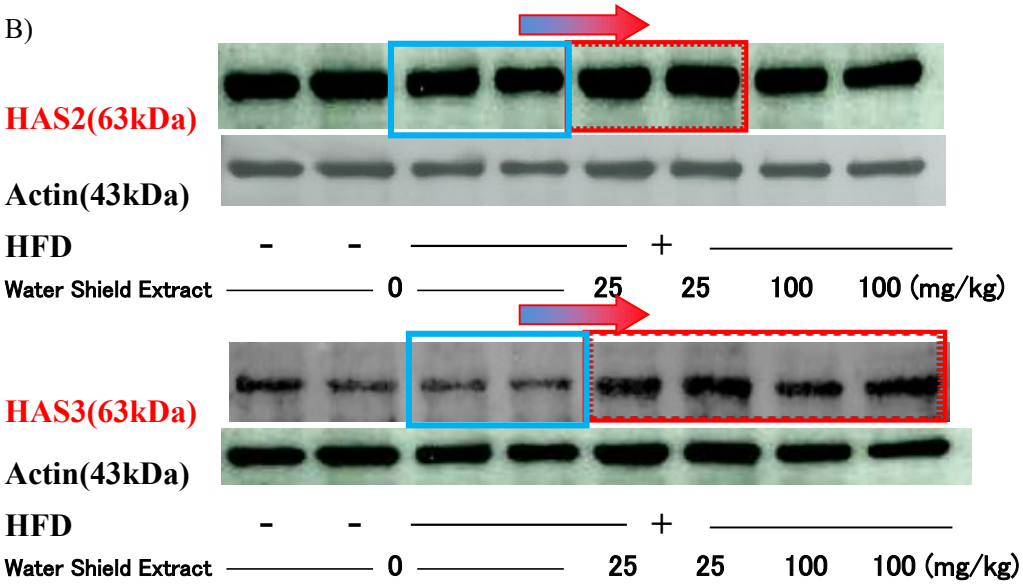
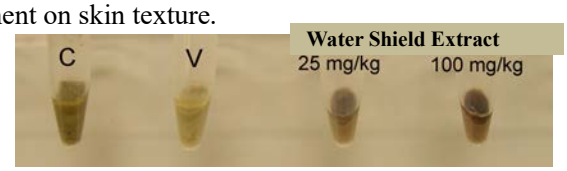


Fig. 12 The Effect of Water Shield Extract on the expression of hyaluronan synthase (HAS) in mice fed with high lipid diet.
A) mRNA expression, mean±SE (n=5-7), B) Protein expression

v. Improvement of intestinal flora

In view of the rich content of polysaccharides in Water Shield Extract, it has been suggested that Water Shield Extract may benefit in the regulation of intestinal flora upon excessive fat consumption. The feces of mice fed with high lipid diet were collected for analysis of indole content. As showed in Fig. 13, color of feces improved in group of mice consuming Water Shield Extract. Meanwhile, harmful substances of ammonia and indole compounds similarly decreased significantly. Above findings confirmed that Water Shield Extract is beneficial in regulating the intestinal flora hence improvement on skin texture.



By high lipid diet, the color of feces changed to whitish (V). Water Shield Extract improved the color to that of control (C).

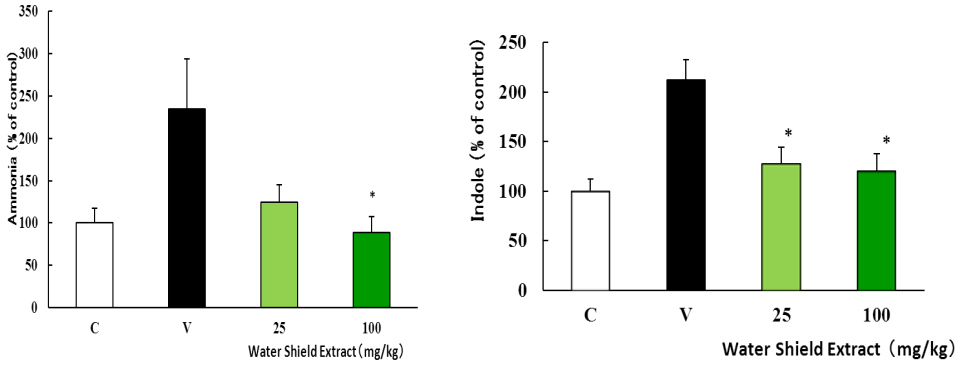


Fig. 13 The Effect of Water Shield Extract on Intestinal Flora of Mice fed with high lipid diet. Upper photo: change in feces, lower graph: ammonia (left) and indole contents. Mean± SE (n=5-7), C: control (normal diet), V: vehicle (high lipid diet), *: $p<0.05$ vs. V.

5. Improvement on Metabolic Syndrome

In collaboration with Harvestch Inc. and Akita Prefecture Food Research Center, the research on the effect of Water Shield Extract on Metabolic Syndrome was introduced. Mice (C57BL/6) were given access to high lipid diet feed containing Water Shield Extract (1%) continuously for 2 weeks. Metabolic syndrome related parameters (e.g. body weight, blood profile, visceral fat) were measured before and after the experiment. Results showed that there is no change in body weight. However, blood cholesterol and visceral fat content has reduced (Fig. 14). In addition, there is significant reduction in blood sugar level, control: 231 ± 20 mg / dL vs. Water Shield Extract: 191 ± 22 mg / dL observed.

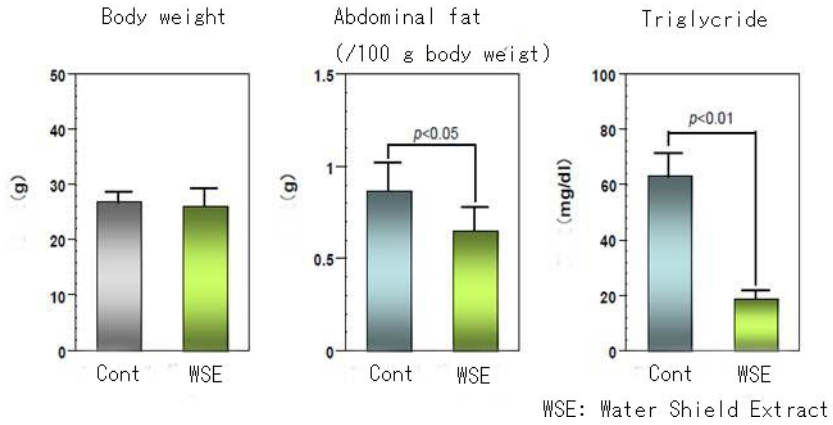


Fig. 14. The Effect of Water Shield Extract on Metabolic Syndrome in mice fed with high lipid diet.

Next, using hepatocyte cell lines, HepG2, oleic acid as source of lipid and Water Shield Extract (100μg/mL) was added to the sample of cell lines and culture for 2 days. The mRNA expression of molecular compounds involved in fat metabolism was evaluated. As showed in Table 2.; all types of cholesterol and triglycerides level were reduced.

Table 2. Effect of water shield extract on lipid accumulation in HepG2 cells induced by oleic acid

	Triglyceride (μg/10^6 cells)				Cholesterol (μg/10^6 cells)			
	Total	VLDL	LDL	HDL	Total	VLDL	LDL	HDL
Normal	7.3**	0.8**	3.8**	2.7	3.6	0.5	1.0	2.1**
Control (oleic acid)	12.6	2.0	7.2	3.4	3.1	0.5	1.2	1.4
H_2O extract	3.9**	0.2**	2.9**	0.8**	0.6**	0.1**	0.3**	0.2**
EtOH extract	1.9**	0.2**	0.9**	0.8**	0.3**	0.0**	0.2**	0.1**

Mean (n=4), **: $p<0.01$.

Meanwhile, expression of the molecular compounds **FAS** (sterol fatty acid synthase – involved in the synthesis of fatty acid), **SREBP1c**(sterol regulatory element binding protein 2 – involved in cholesterol synthesis) and **SREBP2, HMGCS-1** (HMG-CoA synthase-1) were down-regulated suggesting that Water Shield Extract lowers elevated cholesterol level. In addition, mRNA expression of **APO A-1** (apolipoprotein A-1 which is coded for lipid metabolism) and **MTTP**

(microsomal triglyceride transfer protein large subunit) were similarly down-regulated (Fig. 15). Above findings suggested that Water Shield Extract prevent cholesterol and triglyceride synthesis thus prevent metabolic syndrome.

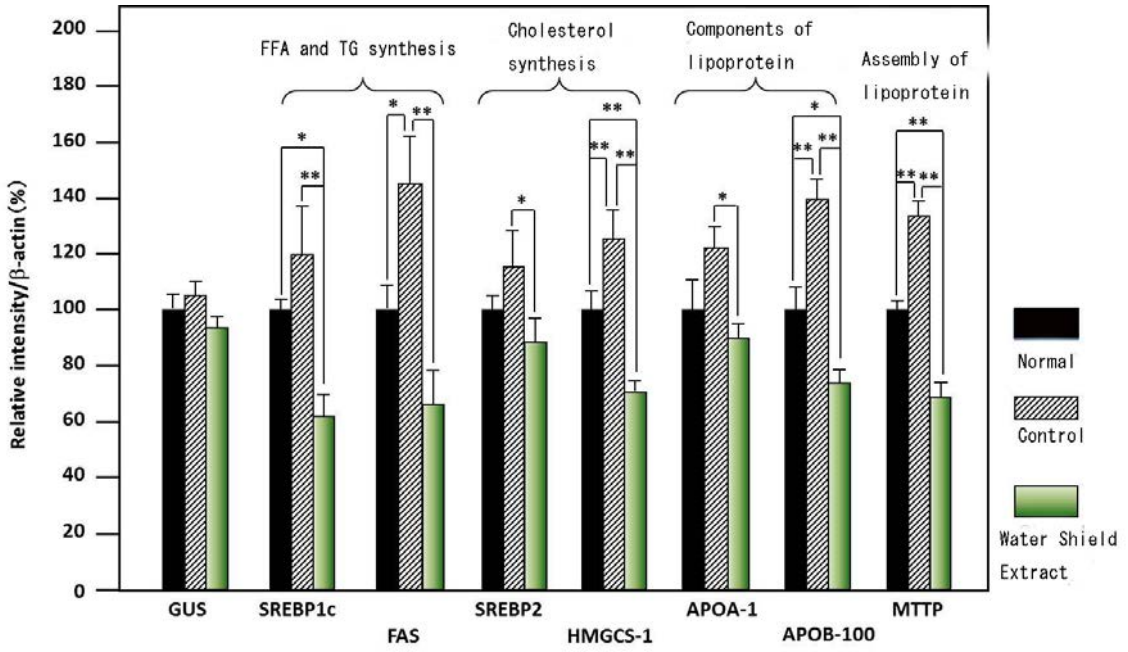


Fig. 15. The Effect of Water Shield Extract on the mRNA of molecular compounds responsible for lipid metabolism in mice fed with high lipid diet. (mean with the SE, n=4)

Fig. 16 showed the changes of cholesterol profile after **12**-week ORAL consumption of Water Shield Extract powder (2.5g). Oral consumption of Water Shield Extract reduced the size and concentration of LDL-cholesterol , therefore, lower the risk of atherosclerosis.

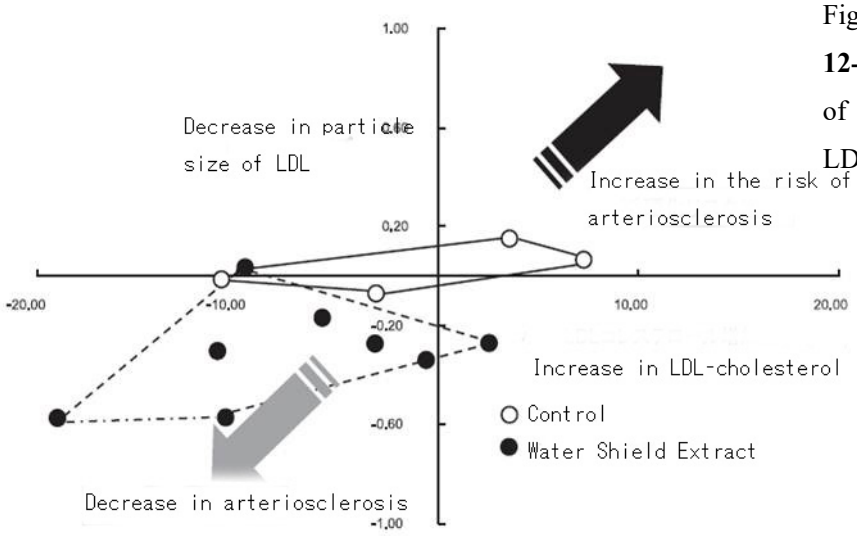


Fig. 16. The effect of **12**-week oral consumption of Water Shield Extract on LDL-cholesterol changes.

6. Human Monitor Test on Water Shield Extract

i. The Effect of ORAL consumption of Water Shield Extract on Obesity parameter on MALE

A human monitor test was conducted on 11 male volunteers to examine the oral effect of Water Shield Extract on obesity parameters.

Protocol:

Test subjects: 11 male volunteers

Sample: Water Shield Extract-P 150mg

Dosage: Once daily after breakfast for 3 weeks

Analysis: percentage of body fat, blood triglyceride level, visceral fat index, thickness of subcutaneous fat, waist measurement measured before and after the test.

As showed in Table 3, body fat percentage, visceral fat index, subcutaneous fat, waist measurement and blood triglyceride level decreased after 3 weeks oral consumption of Water Shield Extract-P 150 mg.

Table 3. Change in parameters in male subject by Water Shield Extract

	Before	After
Body weight（kg）	74.2±4.2	74.4±4.2
Body fat ratio（%）	25.1±1.9	24.5±1.9↓
BMI (kg/m^2)	25.5±1.2	25.6±1.2
Abdominal fat index	109.5±15.0	102.3±13.5↓
Subcutaneous fat (abdomen, cm)	3.1±0.3	2.2±0.2 $p<0.05$ ↓
Subcutaneous fat (arm, cm)	2.6±0.2	2.0±0.2↓
West（cm）	90.4±3.1	88.6±3.0↓
TG（mg/dL）	121±16	111±14↓

Mean±SE（n=11）

In addition, Fig. 17 showed that thickness of subcutaneous fat was noticeably reduced. In conclusion, oral consumption of Water Shield Extract reduced visceral fat and subcutaneous fat.

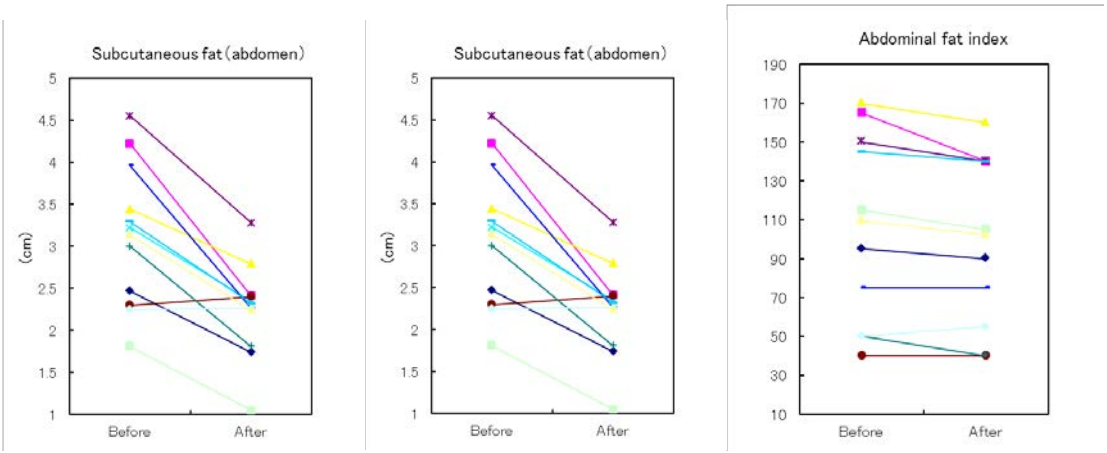
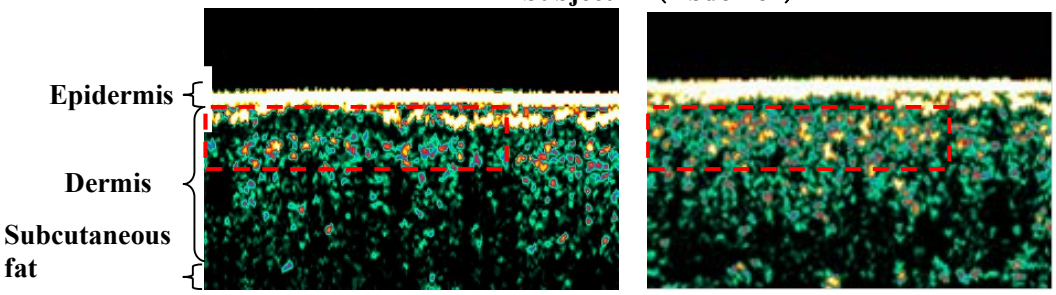


Fig. 17. Change in subcutaneous and abdominal fats by Water Shield Extract

Next, Fig. 18, showed ultrasound images of skin dermis before and after 3-week oral consumption of Water Shield Extract using DermaLab ultrasound imaging system. Water Shield

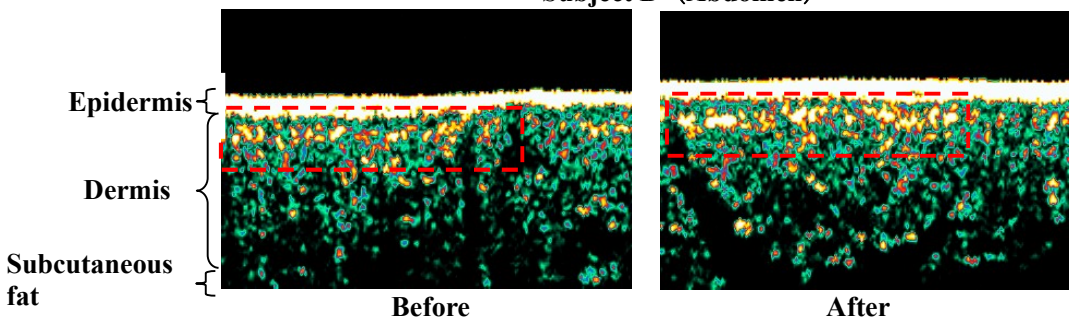
Extract reduced fat penetrate into the dermal layer while increase in pale yellowish region (high density region) indicate increased in dermal collagen. Water Shield Extract effectively reduced visceral fat and subcutaneous fat.

Subject A（Abdomen）



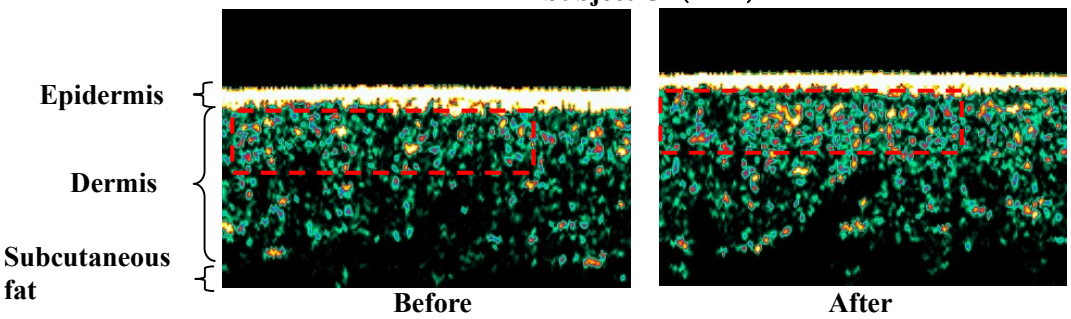
Subcutaneous fat in upper dermis (the black area) can be seen before the treatment. After 3 week oral consumption of Water Shield Extract, the area decreased.

Subject B（Abdomen）



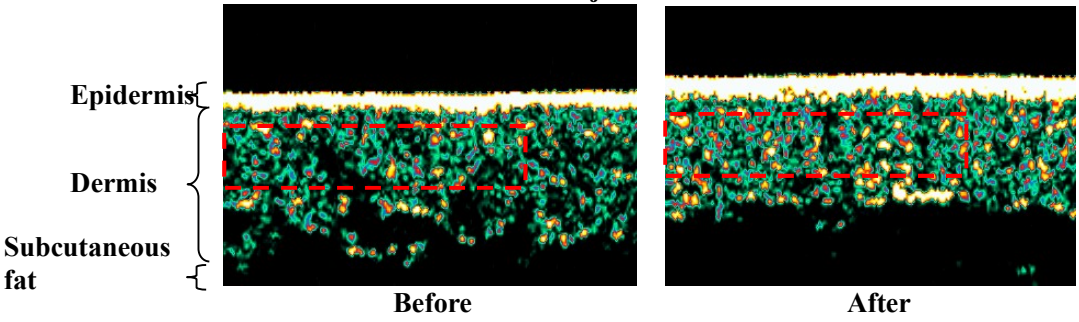
High density region of upper dermis (pale yellow area) increases after 3 week oral consumption of Water Shield Extract, it is suggestive that collagen is increased.

Subject C（Arm）



Similar to Test subject A, subcutaneous fat of upper dermis reduced after 3 week oral consumption of Water Shield Extract.

Subject D（Arm）



Similar to Test subject A, subcutaneous fat of upper dermis reduced after 3 week oral

consumption of Water Shield Extract.

Fig. 18. Ultrasound images of dermis of the hip and upper before and after oral consumption of Water Shield Extract.

ii. The Effect of ORAL consumption of Water Shield Extract on Obesity parameter , Cellulite and Cosmetics effect on FEMALE

a) Obesity Parameter

Similar human monitor test was conducted on 7 FEMALE volunteers to examine the oral effect of Water Shield Extract on obesity parameters, cellulite and Cosmetics appearance.

Protocol:
Test subjects: 7 Female volunteers
Sample: Water Shield Extract-P 150mg
Dosage: Once daily after breakfast for 3 weeks
Analysis: percentage of body fat, blood triglyceride level, visceral fat **index**, thickness of subcutaneous fat, waist measurement and cellulite was measured before and after the test.

As showed in Table 4, percentage of body fat, visceral fat index and hip circumference decreased after 3 week oral consumption of Water Shield Extract-P. Above findings clearly indicated the oral effect of Water Shield Extract on reducing visceral fat and subcutaneous fat.

Table 4. The oral effect of Water Shield Extract on obesity parameters of females

	Before	After
Body weight（kg）	52.8±2.5	53.2±2.6
Body fat ratio （%）	28.7±1.5	27.4±2.1↓
BMI （kg/m^2）	20.7±0.7	20.9±0.8
Abdominal fat index	38.6±3.4	36.4±2.8↓
Subcutaneous fat (abdomen, cm)	2.6±0.2	2.6±0.2
Subcutaneous fat (arm, cm)	2.7±0.1	2.7±0.1
Subcutaneous fat (under hip, cm)	1.8±0.3	1.8±0.3
Hip size （cm）	92.1±1.7	91.7±1.7↓

Mean±SE （n=10）

Next, Fig. 19 showed an ultrasound image of the dermis of upper arm after the oral consumption of Water Shield Extract. There is a reduction in the fat penetrating into the dermis (the black region within the red dotted line) observed while the collagen score increased.

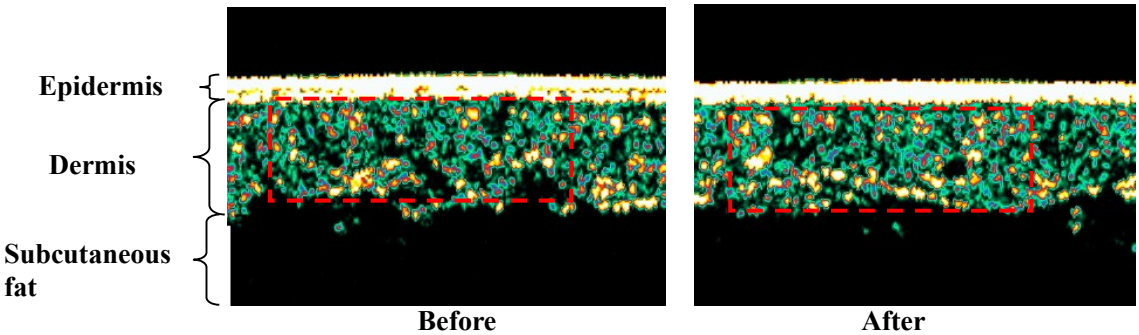
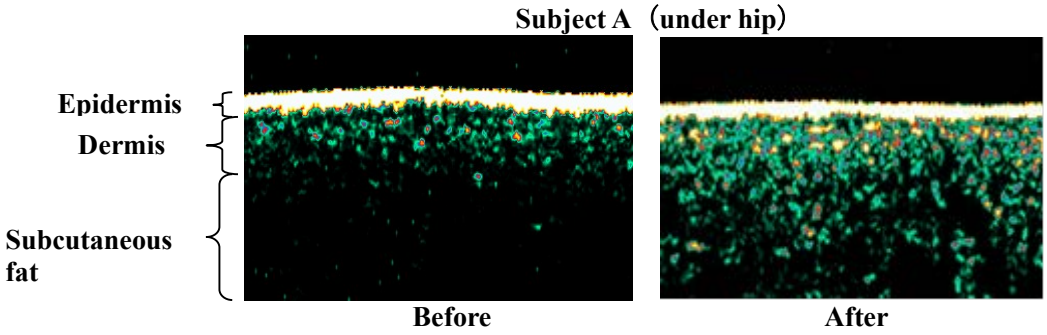


Fig. 19 Ultrasound images of the dermis of upper arm before and after oral consumption of Water Shield Extract.

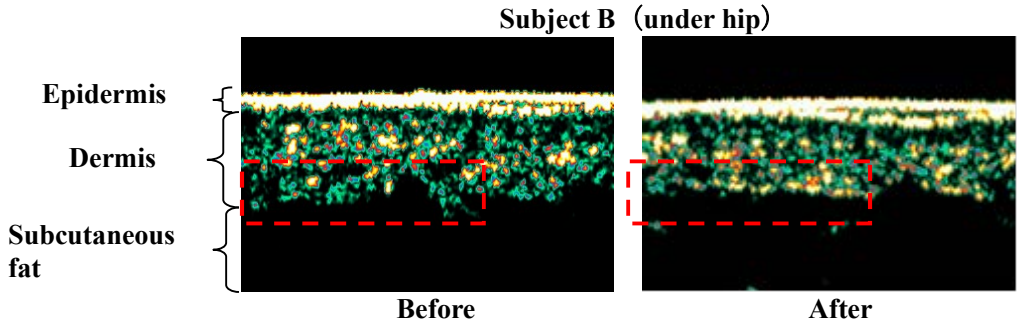
b) Subcutaneous fat of the hip and cellulite

The ultrasound images of the dermis if the hip is shown in Fig. 20. It is clearly noticed that in Test subject A, the subcutaneous fat (black region) area is reduced while dermis grew after 3 week oral consumption of Water Shield Extract. In test subject B, the hidden cellulite observed as unevenness at the bottom of the dermis layer is similarly reduced. Fig. 20B showed that the quantity of cellulite as well as the depth of cellulite is reduced. It is suggestive that Water Shield Extract is anti-cellulite.

A)

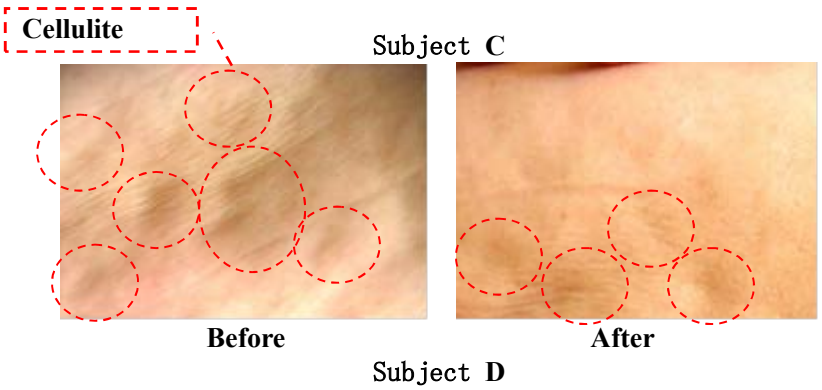


The amount of subcutaneous fat (black region) was reduced after oral consumption of Water Shield Extract.



Hidden cellulite observed as unevenness at the bottom of the dermis layer, within the dotted line) is reduced after oral consumption of Water Shield Extract.

B)



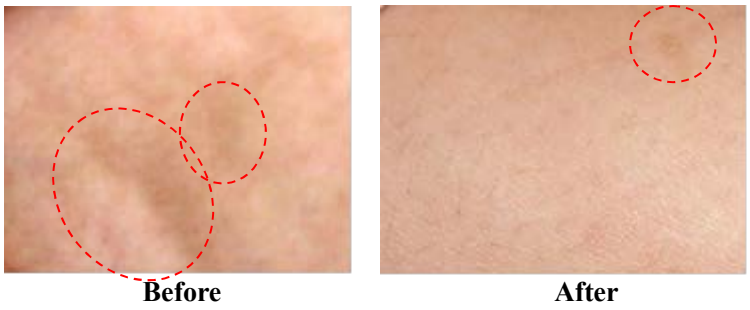
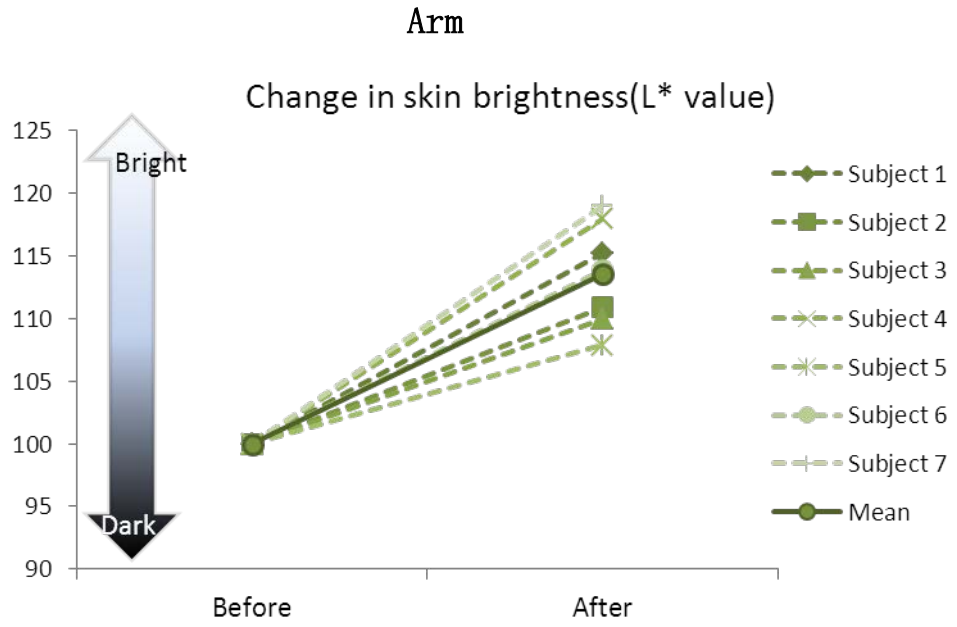


Fig. 20 Ultrasound images of dermis of the hip (A) and cellulite (B) after oral consumption of Water Shield Extract.

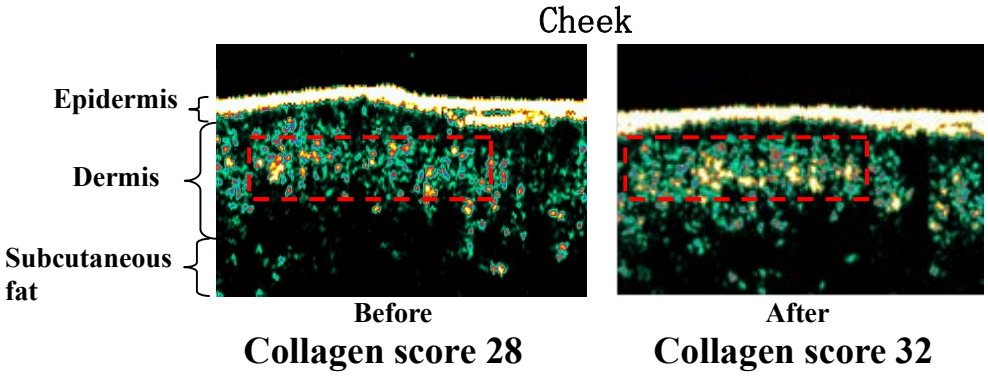
c) Beauty index

In addition to above findings, brightness of the inner side of skin of the upper arm was quantified as beauty index in the human monitor test. As illustrated in Fig. 21A, the L* value which indicates brightness of the skin increased in all test subjects after 3-week consumption of Water Shield Extract. Besides, ultrasound images showed that the high density area (red to yellow region, high density area of collagen) is expanded, therefore collagen score increased.

A)



B)



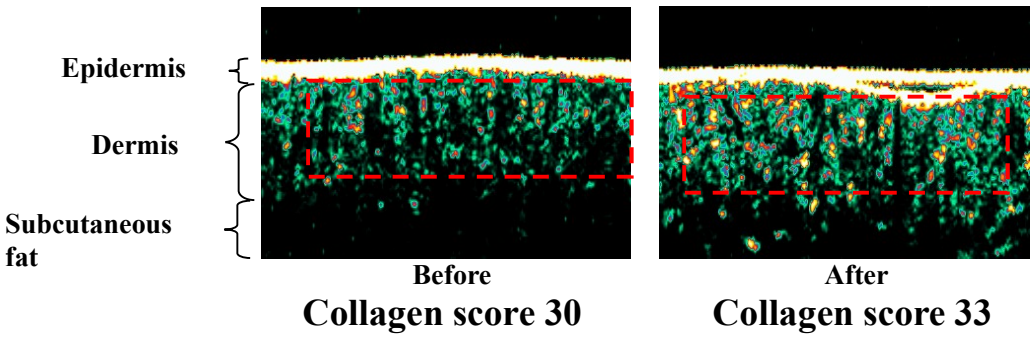


Fig. 21A The oral effect of Water Shield Extract on the brightness of the inner side of skin of upper arm

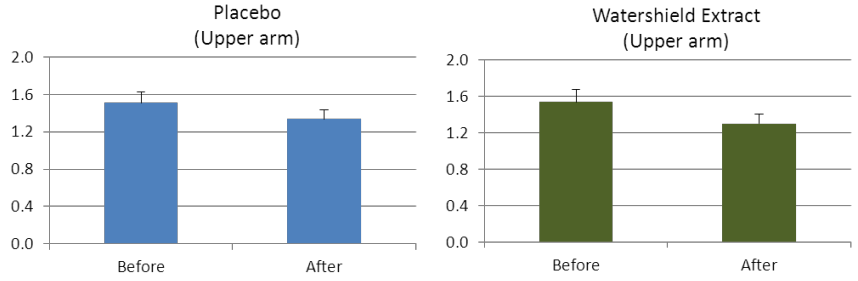
Fig. 21B Ultrasound images of the cheek before and after oral consumption of Water Shield Extract.

iii. Topical Effect of Water Shield Extract on cellulite and subcutaneous fat in female

a) Subcutaneous fat and cellulite

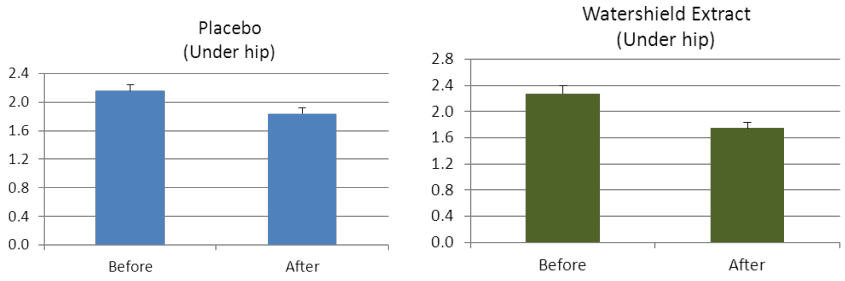
The topical effect of Water Shield Extract on cellulite and subcuteneous fat was examined. Topical application of gel containing Water Shield Extract-LC 10% was applied on 5 female volunteers on the thigh and lower hip for 2 weeks. Subcutaneous fat thickness of upper arm and thigh was measured before and after topical application of gel containing Water Shield Extract-LC 10%. As showed in Fig. 22 A,B, thickness of subcutaneous fat of upper arm and thigh reduced upon comparison with placebo. Ultrasound images showed that hidden cellulite (unevenness at the bottom of the dermis, within red-dotted line) is reduced and collagen score increased (Fig. 22C) after 2-week topical application of gel containing Water Shield Extract-LC 10%. In addition, Fig. 22D showed that the cellulite appearance on skin is reduced. Above findings clearly indicated the anti-cellulite effect of Water Shield Extract.

A)



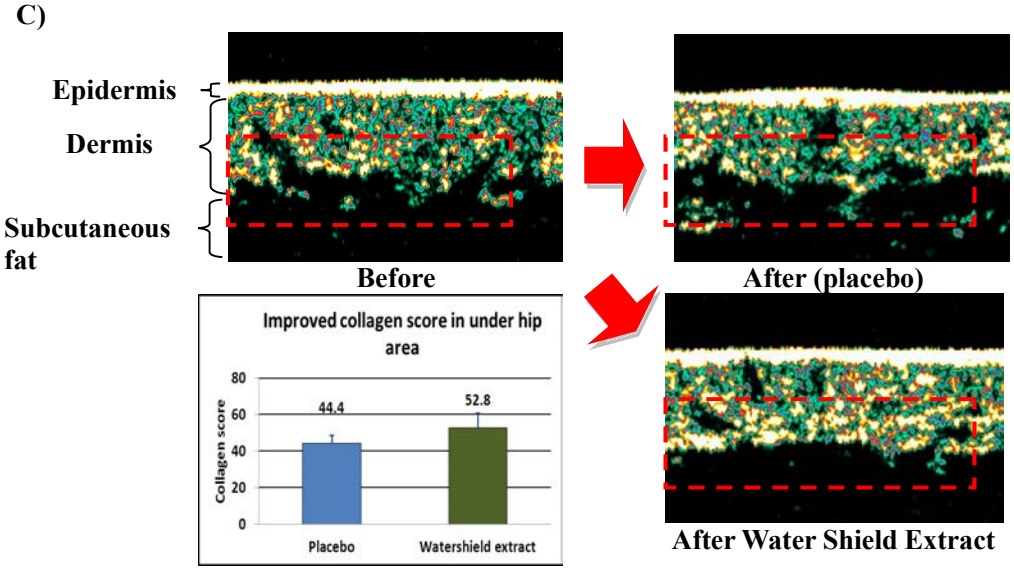
Mean±SE

B)



Mean±SE

C)



D)

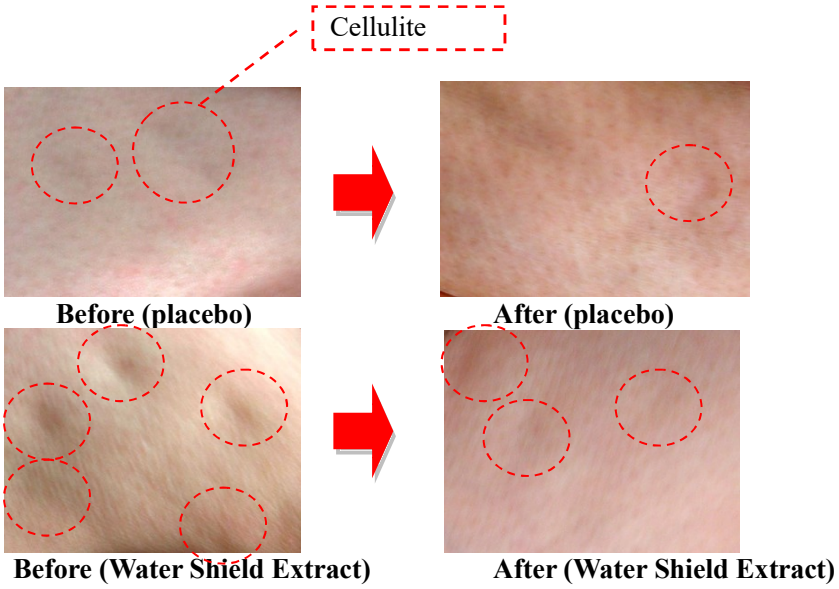


Fig. 22 The Topical Effect of Water Shield Extract-LC
22A, 22B: thickness of subcutaneous fat
22C: ultrasound images & collagen score
22D: changes on cellulite

b. Beauty index

Elasticity of skin of upper arm was measured as beauty index and compared with placebo before and after the topical application of Water Shield Extract-LC gel. As illustrated in Fig. 23, elasticity of skin of upper arm improved compared with placebo. Meanwhile, there is no difference in the collagen score upon comparison with placebo, hence it is suggestive that improvement on skin elasticity is non-collagen related.

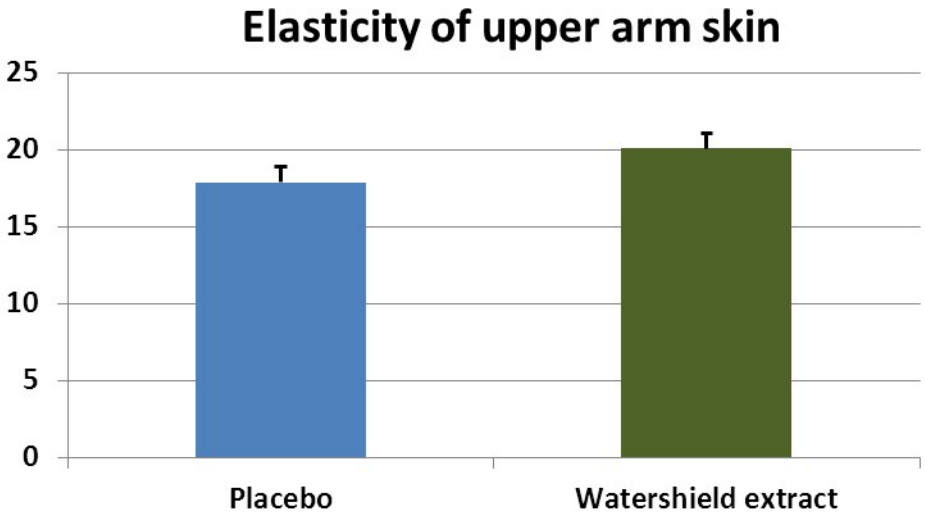


Fig. 23 The topical effect of Water Shield Extract-LC gel on skin elasticity of upper arm. Mean with the SE

7. Product Stability

i. Heat stability

The heat stability of Water Shield Extract-P was evaluated. As illustrated in Fig. 24, content of polyphenol of Water Shield Extract remain stable after continuous heating at 120°C for 1 hour. It is highly stable at normal food processing temperature.

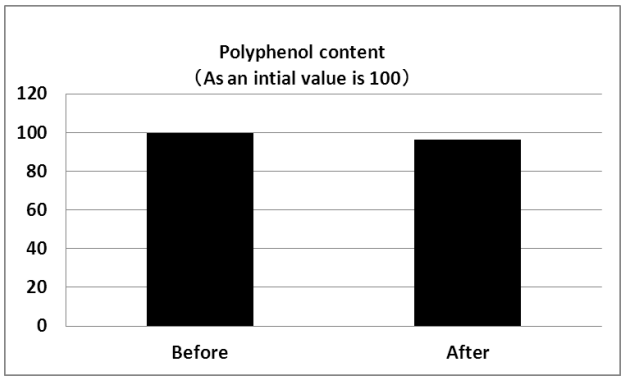


Fig. 24 The heat stability of Water Shield Extract-P.

ii. pH stability

The pH stability of Water Shield Extract was examined. Water Shield Extract-WSP was solubilized in water and adjusted to different pH condition was left for storage at room temperature with light for 1 week. Polyphenol content of Water Shield Extract-WSP in solution was quantified after 1 week and color of solution was observed. At the end of 1 week, no color changes observed in all samples containing Water Shield Extract-WSP at different pH condition. Similarly, the polyphenol content of Water Shield Extract remained stable at all pH ranges.

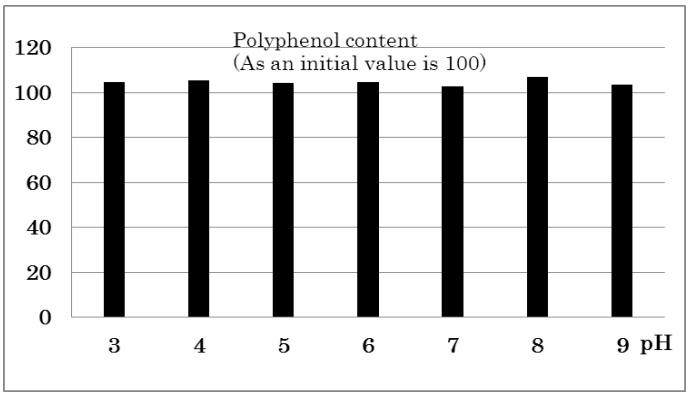


Fig. 25 The pH stability of Water Shield Extract-WSP

8. Nutrition Profiles

Analyzed item	P	WSP	Method
Water	6.6 g/100 g	6.6 g/100 g	Heating drying method under normal pressure
Protein	0.9 g/100 g	0.9 g/100 g	Kjeldahl method, nitrogen protein conversion factor: 6.25
Fat	3.2 g/100 g	3.2 g/100 g	Acid decomposition method
Ash	1.4 g/100 g	1.4 g/100 g	Direct incineration method
Carbohydrate	83.5 g/100 g	83.5 g/100 g	Refer note 1
Energy	375 kcal/100 g	375 kcal/100 g	Refer note 2
Fiber	4.4 g/100 g	4.4 g/100 g	Prosky's method
Sodium	281 mg/100 g	281 mg/100 g	Atomic absorption spectrophotometory

The nutritional information of Water Shield Extract was analyzed according to the standard in nutrition labeling (April 26, 1999; No 13 Eishin)
Note 1: Calculation: 100-(water + protein + fat + ash)
Note 2: Energy conversion factor: Protein 4, fat 9, sugar 4; dietary fiber 2
Test trustee: SUNATECH / Date of analysis: Sept 28, 2012 / Test No.: 120918022-001-01

9. Product Stability

i. Residual Agricultural Chemicals

As confirmed by test trustee, analysis results of **Water Shield Extract** is conformed to the regulation stipulated in the Food Sanitation Act. on 535 residual agricultural compounds.

Test trustee：Masis Co. Ltd.
Data：Sept 10, 2012
Report No.：56984

ii. Acute toxicity (LD_{50})

Acute Toxicity test was conducted according to the Guidelines for Single-Dose Toxicity Tests for Pharmaceutical Products where **Water Shield Extract** 2000mg/kg was orally given to starved ICR mice (male & female ddy, 6 weeks old, weight ~30g) for 14 days. No abnormalities and fatal event observed at 2000mg/kg. No abnormalities of organs observed under macroscopic examination upon autopsy. Thus, LD_{50} of Water Shield Extract is deduced to be >2000mg/kg.

10. Recommended Daily Dose

Description	Recommended Daily Dose	Product
FOOD	150mg	Water Shield Extract-P, -WSP
COSMETICS	0.1-0.6%	Water Shield Extract-P, -WSP
	1-10%	Water Shield Extract-LC

11. Application

	Applications	Claims	Examples
Food	Nutritional Supplement Beauty Food Anti-cellulite	Diet, supplement, beauty, regulation of intestinal flora, anti-cellulite, collagen production, antioxidant, hyaluronic acid production	Beverages Hard & soft capsules, tablets Candies, chewing gums, chocolates, wafers, jellies Ham, sausage, etc.
Cosmetics	Skin care Cosmetics		Lotions, toner, serum, rinse, treatment care, pack, body gel etc.

12. Packaging

Product	Packing	Weight
Water Shield Extract-P Water Shield Extract-WSP	Interior packing: Aluminum bag Exterior packaging: Cardboard	1kg 5kg
Water Shield Extract-LC	Interior packing: Bottle Exterior packing: Cardboard	1kg
	Interior packing: Cubic polyethylene container Exterior packing: Cardboard	5kg

13. Storage

Store in a cool, dry and dark place. Avoid heat and places with high humidity.
It is recommended to finish using the product once open as it is highly hygroscopic. Otherwise, desiccant bag is recommended to be inserted for storage purpose.

14. Expression

(FOOD)

Water Shield Extract-P, -WSP
Expression: Water Shield Extract, Dextrin

(Cosmetics)

Water Shield Extract-PC,-WSPC
INCI: SODIUM STARCH OCTENYLSUCCINATE (and) BRASENIA SCHREBERI LEAF EXTRACT
Water Shield Extract-LC
INCI: PROPANEDIOL (and) WATER (and) BRASENIA SCHREBERI LEAF EXTRACT

PRODUCT STANDARD

WATERSHIELD EXTRACT-P (FOOD)

This product is extracted with aqueous ethanol from the leaves of watershield (*Brasenia schreberi*).

Appearance	Pale yellowish brown to gray-yellowish green powder with slightly unique scent.	
Polyphenols	Min. 5.0 %	(Folin-Denis method)
Junsainoside A	Min. 0.01 %	(HPLC)
Polysaccharides (Originated from watershield)	Min. 2.5 %	(Phenol-sulfuric acid method)
Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105ºC, 2 hr)
Purity Test		
(1) Heavy Metals (as Pb)	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	Ingredient	Content
	Watershield Extract	17 %
	Modified Starch	83 %
	Total	100 %
Expiry date	2 years from date of manufacturing.	
Storage	Store it in a cool, dry, ventilated area with desiccant. Keep it away from high temperature and sunlight, and store it in a closed container.	

PRODUCT STANDARD

WATERSHIELD EXTRACT-WSP (FOOD)

This water-soluble product is extracted with aqueous ethanol from the leaves of water shield Brasenia schreberi J.F.Gmelin.

Appearance	Pale yellowish brown to gray-yellowish green powder with slightly unique scent.	
Polyphenols	Min. 5.0 %	(Folin-Denis method)
Junsainoside A	Min. 0.01 %	(HPLC)
Polysaccharides (Originated from watershield)	Min. 2.5 %	(Phenol-sulfuric acid method)
Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105ºC, 2 hr)
Purity Test		
(1) Heavy Metals (as Pb)	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

Ingredient	Content
Watershield Extract	17 %
Modified Starch	83 %
Total	100 %

Expiry date 2 years from date of manufacturing.

Storage Store it in a cool, dry, ventilated area with desiccant. Keep it away from high temperature and sunlight, and store it in a closed container.

PRODUCT STANDARD

WATERSHIELD EXTRACT-PC (COSMETICS)

This product is extracted with aqueous ethanol from the leaves of watershield (*Brasenia schreberi*).

Appearance	Pale yellowish brown to gray-yellowish green powder with slightly unique scent.	
Polyphenols	Min. 5.0 %	(Folin-Denis method)
Junsainoside A	Min. 0.01 %	(HPLC)
Polysaccharides (Originated from watershield)	Min. 2.5 %	(Phenol-sulfuric acid method)
Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105ºC, 2 hr)
Purity Test		
(1) Heavy Metals (as Pb)	Max. 20 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

Ingredient	Content
Sodium Starch Octenylsuccinate	83 %
Brasenia Schreberi Leaf Extract	17 %
Total	100 %

Expiry date	2 years from date of manufacturing.
Storage	Store it in a cool, dry, ventilated area with desiccant. Keep it away from high temperature and sunlight, and store it in a closed container.

PRODUCT STANDARD

WATERSHIELD EXTRACT-WSPC (COSMETICS)

This product is extracted with aqueous ethanol from the leaves of watershield (*Brasenia schreberi*).

Appearance	Pale yellowish brown to gray-yellowish green powder with slightly characteristic odor.	
Polyphenols	Min. 5.0 %	(Folin-Denis method)
Junsainoside A	Min. 0.01 %	(HPLC)
Polysaccharides (Originated from watershield)	Min. 2.5 %	(Phenol-sulfuric acid method)
Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105ºC, 2 hr)
Purity Test		
(1) Heavy Metals (as Pb)	Max. 20 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

Ingredient	Content
Sodium Starch Ooctenylsuccinate	83 %
Brasenia Schreberi Leaf Extract	17 %
Total	100 %

Expiry date	2 years from date of manufacturing.
Storage	Store it in a cool, dry, ventilated area with desiccant. Keep it away from high temperature and sunlight, and store it in a closed container.

PRODUCT STANDARD

WATERSHIELD EXTRACT-LC (COSMETICS)

This product is extracted with aqueous ethanol from the leaves of watershield (*Brasenia schreberi*) and dissolved in aqueous propanediol.

Appearance Yellowish brown to brown liquid with slightly unique scent.

Certification Test Dissolve 30 μl of this product in 3.5 ml water. Add
Polyphenols 0.2 ml Folin-Denis reagent into the solution followed by 0.4 ml saturated Na_2CO_3. The solution will be blue.

Purity Test

(1) Heavy Metals (as Pb)	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

Ingredient	Content
Propanediol	70 %
Water	29 %
Brasenia Schreberi Leaf Extract	1 %
Total	100 %

Expiry date 2 years from date of manufacturing.

Storage Store it in a cool, dry, ventilated area with desiccant. Keep it away from high temperature and sunlight, and store it in a closed container.

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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ORYZA OIL & FAT CHEMICAL CO., LTD.