

Anti-Adipogenic Polyphenols of Water Shield Suppress TNF- α -Induced Cell Damage and Enhance Expression of HAS2 and HAPB2 in Adiponectin-Treated Dermal Fibroblasts

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Abstract

Water shield (*Brasenia schreberi* J. F. Gmel) is an aquatic plant cultivated in Northern Japan, from which we have isolated polyphenolic compounds including a novel flavonol glycoside, *junsainoside* A. Recently, dermal fibroblasts have reported to be affected by adipocytokines from subcutaneous adipocytes. Hence, we evaluated the effects of polyphenolic compounds from water shield on fat accumulation, adipocytokine release and adipocytokine-induced fibroblast status. In differentiated 3T3-L1 adipocytes, ethyl gallate, caffeoyl glucose, hypolaetin 7-O-glucoside, kaempferol and junsainoside A significantly suppressed lipid accumulation at 10 μ M. In addition to these constituents, ferulic acid, kaempferol 3-O-(6"-galloyl) glucoside, quercetin 3-O-(6"-galloyl) glucoside, quercetin 3-O-glucoside and gossypetin suppressed the lipid accumulation in human subcutaneous adipocytes. Ethyl gallate and junsainoside A also enhanced adiponectin production from subcutaneous adipocytes without induction of TNF- α release. On the other hand, ethyl gallate and quercetin recovered from TNF- α -induced fibroblast damage. Moreover, ferulic acid, quercetin and kaempferol enhanced the expression of hyaluronan binding protein 2 and hyaluronan synthase 2 in adiponectin-treated fibroblasts. These results suggested that water shield contains various types of polyphenolic compounds with anti-adipogenic activity, some of which protect dermal fibroblasts from TNF- α damage and probably enhance hyaluronan production. It is assumed that polyphenols in water shield maybe suppress subcutaneous fat accumulation and maintain dermal fibroblast and hyaluronan-related status.

Keywords: Water shield; Junsainoside A; Adipocyte; TNF- α ; Adiponectin; Hyaluronan

Abbreviations: TNF: Tumor Necrosis Factor- α , β ; WSE: Water shield extract, DEPT: Distortionless enhancement by polarization transfer, HMQC: Heteronuclear multiple quantum coherence spectroscopy, HMBC: Heteronuclear multiple bond connectivity, RIPA: Radioimmunoprecipitation assay, HAS: Hyaluronan synthase, HABP: Hyaluronan binding protein, HRP: Horseradish peroxidase, D-MEM: Dulbecco's modified essential medium, FCS: Fetal calf serum, DMSO: Dimethyl sulfoxide, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Introduction

Water shield (*Brasenia schreberi* J. F. Gmel, Cabombaceae) is an aquatic plant that grows in Asia, Australia, Africa and North America. As the surfaces of its leaves and buds are covered with mucus consisting of polysaccharide [1], the texture is similar to gelatine. In Japan, water shield (*junsai* in Japanese) is cultivated as a vegetable and the young buds are used as a food ingredient. On the other hand, the leaves have been used as stomachic in China; however, the constituents of water shield have not been well studied. Only quercetin 7-O-glucoside and gallic acid have been reported as principal polyphenols [2]. In terms of the biological activity of the extract from water shield, anti-oxidative [2], anti-inflammatory [2] and hypolipidemic activities [3] have been reported. Considering this background, we performed isolation of the constituents of water shield leaves and identified various types of polyphenol including a new flavonol glycoside named *junsainoside* A.

A number of polyphenols have been reported to suppress visceral fat accumulation in adipocytes. 3T3-L1 preadipocytes [4] and primary cultured visceral adipocytes [5] have been frequently used for the assay. These results indicate the efficacy of polyphenols against obesity-related insulin resistance, dyslipidemia and arteriosclerosis. However, from the perspective of dermatology, the effect of polyphenols on subcutaneous fat accumulation has not been well studied. Recently, the

relationship between subcutaneous adipocytes and dermal fibroblasts has been focused on. Subcutaneous fat exists in the hypodermis and releases adipocytokines including tumor necrosis factor (TNF) - α and adiponectin [6]. TNF- α is also released from dermal inflammatory cells and induces fibroblast apoptosis [7]. On the other hand, adiponectin enhances hyaluronan and collagen production from fibroblasts [8]. Thus, TNF- α and adiponectin affect dermal fibroblasts with negative and positive responses and the balance of these adipocytokines is considered to be crucial to maintain stable dermal conditions. Against the above background, we investigated the effect of water shield extract (WSE) and its constituents on lipid accumulation and adipocytokine release in subcutaneous adipocytes. In addition, the effects of the constituents on fibroblasts treated with adipocytokines were evaluated.

Materials and Methods

Preparation of WSE

The leaves of water shield cultivated in Mitane town (Akita prefecture, Japan) were harvested in June 2011. The leaves were air-dried and ground. The powdered leaves (200 g) were then extracted with 30 w/w% ethanol (EtOH, 6 kg) at 50°C for 2 h. The solvent was evaporated to obtain WSE (44.3 g, yield: 22.1%). The polyphenol

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Received May 29, 2014; Accepted August 18, 2014; Published August 20, 2014

Citation: Shimoda H, Nakamura S, Hitoe S, Terazawa S, Tanaka J, et al. (2014) Anti-Adipogenic Polyphenols of Water Shield Suppress TNF- α -Induced Cell Damage and Enhance Expression of HAS2 and HAPB2 in Adiponectin-Treated Dermal Fibroblasts. Nat Prod Chem Res 2: 146. doi:10.4172/2329-6836.1000146

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content determined by the Folin-Ciocalteu method was 27.3% (gallic acid equivalent).

Isolation of the compounds in water shield

Dried powder of water shield (870 g) was extracted with 50 w/w% EtOH (20 kg) at 80°C for 3 h. The solvent was evaporated to obtain 50% EtOH extract (188 g, yield: 21.6%). The extract (100 g) was applied to Diaion[®] HP20 (Mitsubishi Chemical Corporation, Tokyo, Japan) column chromatography (1 kg). The column was eluted with 10, 30, 70 and 100 v/v% methanol (MeOH) sequentially to obtain a 10% MeOH fraction (2.3 g), 30% MeOH fraction (14.2 g), 70% MeOH fraction (29 g) and 100% MeOH fraction (4.7 g). The 10% MeOH fraction (2.3 g) was applied to octadecyl silyl (ODS, Fuji Silysia Chemical Ltd., Kasugai, Japan) silica gel column chromatography (50 g, 10 \rightarrow 20 \rightarrow 30% MeOH) to obtain fractions: fraction (Fr.) 1 (0.79 g), Fr. 2 (0.75 g) and Fr. 3 (0.42 g). Fr. 2 (0.75 g) was purified by HPLC (C₃₀ column (Develosil RPAQUEOUS; Nomura Chemical Co. Ltd., Seto, Japan, 250 \times 20 mm *i.d.*), 10% MeOH) to obtain phenylalanine (**1**, 2 mg, yield: 0.002% from 50% EtOH extract). Fr. 3 (0.42 g) was separated by HPLC (C₃₀ column, 50% MeOH) to obtain *p*-phenylbenzoic acid (**2**, 2 mg, 0.002%) [9]. The 70% MeOH fraction (29 g) was applied to ODS column chromatography (300 g, 40 \rightarrow 50 \rightarrow 60 \rightarrow 70 \rightarrow 100% MeOH) to obtain fractions: Fr. 1 (23.9 g), Fr. 2 (1.4 g), Fr. 3 (1.8 g), Fr. 4 (0.4 g) and Fr. 5 (0.2 g). Fr. 1 (1 g) was

purified by HPLC [ODS column (Inertsil preparative ODS; GL Science, Tokyo, Japan, 250 \times 20 mm *i.d.*, 40% MeOH) and C₃₀ column (60% MeOH)] to obtain ethyl gallate (**3**, 3.2 mg, 0.076%) [10], ferulic acid (**4**, 0.6 mg, 0.014%) and caffeoyl glucose (**5**, 1 mg, 0.024%) [11]. Fr. 2 (1.4 g) was purified by HPLC (ODS column (50% MeOH) and C₃₀ column (50-60% MeOH)) to obtain hypolaetin 7-*O*-glucoside (**6**, 1 mg, 0.001%) [12], kaempferol 3-*O*-(6''-galloyl) glucoside (**7**, 6 mg, 0.006%) [13], quercetin 3-*O*-(6''-galloyl) glucoside (**8**, 8 mg, 0.008%) [14], quercetin 7-*O*-glucoside (**9**, 2 mg, 0.002%) [15], quercetin 3-*O*-glucoside (**10**, 26 mg, 0.026%) [16] and gossypetin (**11**, 7 mg, 0.007%) [17]. Fr. 3 (1.8 g) was purified by HPLC (ODS column (60% MeOH) and C₃₀ column (65-70% MeOH)) to obtain junsainoside A (**12**, 32 mg, 0.032%), quercetin (**13**, 62 mg, 0.062%) and kaempferol 7-*O*-(6''-galloyl) glucoside (**14**, 2 mg, 0.002%) [18]. The 100% MeOH eluent (4.65 g) was applied to ODS column chromatography (100 g, 70 \rightarrow 80 \rightarrow 90 \rightarrow 100% MeOH) to obtain fractions: Fr. 1 (1.62 g), Fr. 2 (0.77 g), Fr. 3 (0.67 g) and Fr. 4 (0.95 g). Fr. 1 (1.62 g) was purified by HPLC (ODS column (70-80% MeOH) and C₃₀ column (70-80% MeOH)) to obtain quercetin (**13**, 84 mg, 0.084%) and kaempferol (**15**, 54 mg, 0.055%). A refractive index detector was used for detection of the peak on HPLC purification. Isolated known compounds were identified by comparison of ¹³C- and ¹H-NMR spectra and mass spectra with reported values or authentic standard samples of **1**, **4**, **13** and **15** (Figure 1).

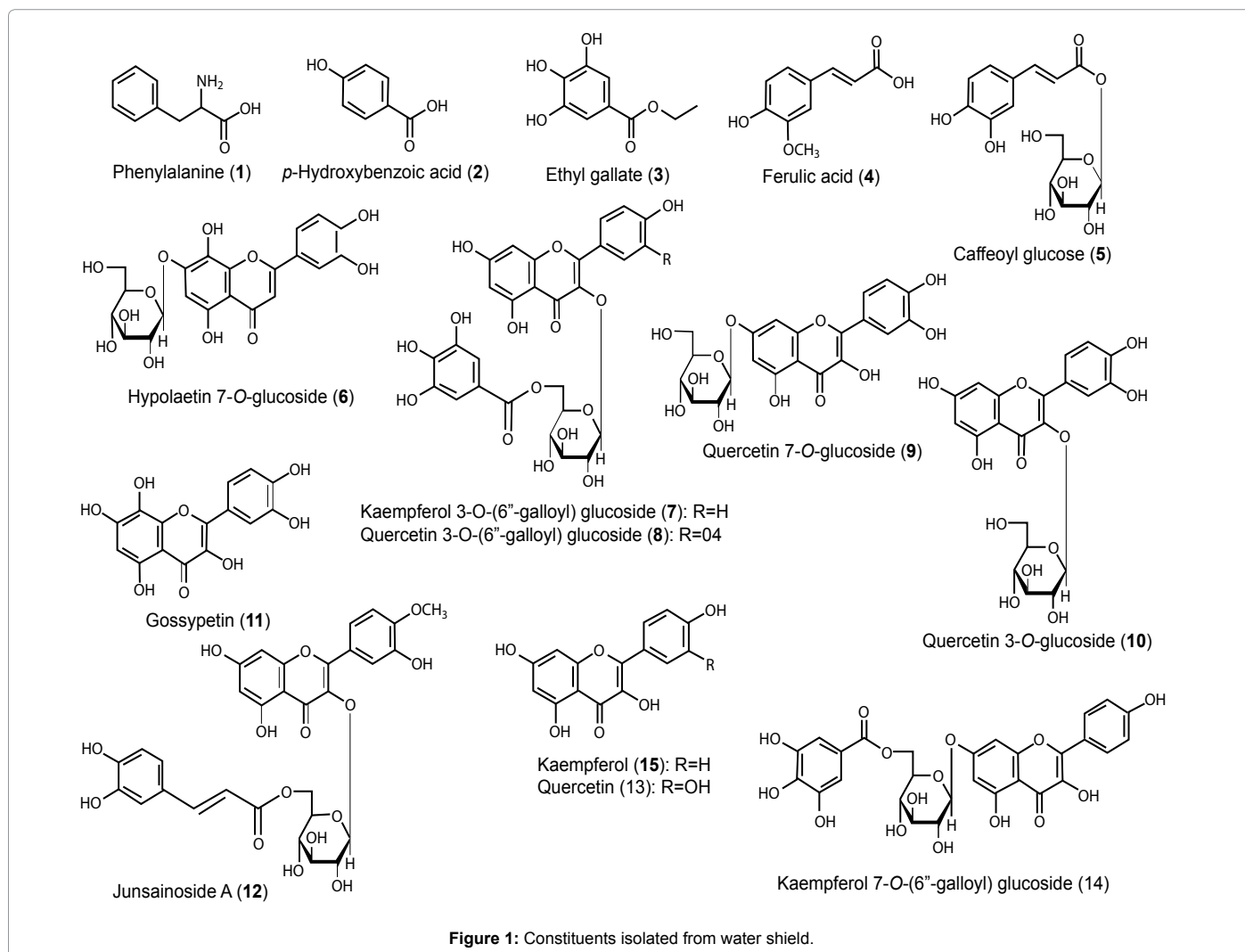
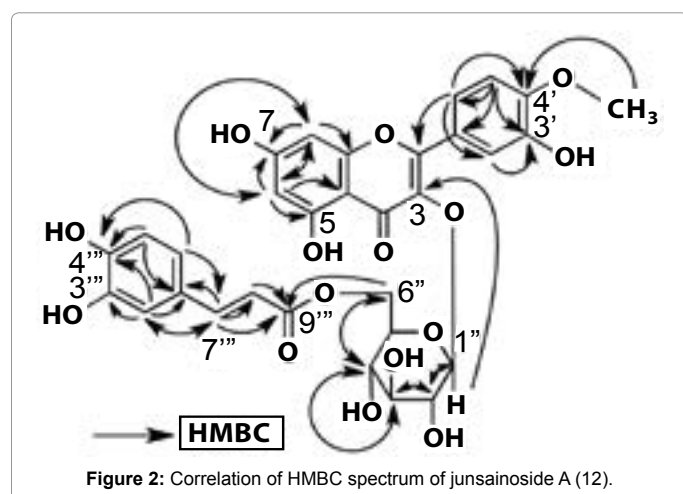


Figure 1: Constituents isolated from water shield.

Structural elucidation of junsainoside A (12)

Junsainoside A (12) was isolated as a pale yellow powder with negative optical rotation ($[\alpha]_D^{21} -52.0^\circ$ in MeOH). The IR spectrum of 12 suggested the presence of hydroxyl (3400 cm^{-1}), α , β -unsaturated ester (1689 cm^{-1}), γ -pyrone (1655 cm^{-1}), aromatic ring (1605 , 1514 cm^{-1}) and ether (1016 cm^{-1}). In the positive-ion matrix-assisted laser desorption/ionization (MALDI)-MS of 12, a quasimolecular ion peak was observed at m/z 663 ($M+Na$) $^+$, and high-resolution (HR) MALDI-MS analysis of the quasimolecular ion peak revealed the molecular formula of 12 to be $C_{31}H_{28}O_{15}$. Acid hydrolysis of 12 with 5% aqueous H_2SO_4 -1,4-dioxane yielded D-glucose, which was identified by HPLC of the tolylthiocarbamoyl thiazolidine derivative. To determine the absolute configuration of glucose of 12, the method reported by Tanaka et al. was used [19]. The 1H -NMR (CD_3OD) and ^{13}C -NMR spectra of 12, which were assigned by various NMR experiments, showed signals assignable to a tamarixetin part [δ 6.07 (1H, d, $J=2.0$ Hz, H-6), 6.25 (1H, d, $J=2.0$ Hz, H-8), 6.80 (1H, d, $J=8.8$ Hz, H-5'), 7.56 (1H, dd, $J=8.8$, 2.0 Hz, H-6'), 7.57 (1H, d, $J=2.0$ Hz, H-2'), a β -D-glucopyranosyl moiety [δ 4.28 (1H, m, 6''-H $_2$), 3.35 (1H, m, 4-H), 3.45 (1H, m, 5-H), 3.43 (1H, m, 3-H), 3.50 (1H, m, 2-H), 5.26 (1H, d, $J=7.3$ Hz, 1''-H)], and a (*E*)-caffeoyl group [δ 6.12 (1H, d, $J=16.0$ Hz, 8'''-H), 6.79 (1H, d, $J=8.3$ Hz, 3'''-H), 6.91 (1H, dd, $J=2.0$, 8.3 Hz, 2'''-H), 7.04 (1H, d, $J=2.0$ Hz, 6'''-H), 7.39 (1H, d, $J=16.0$ Hz, 7'''-H)]. The 1H and ^{13}C NMR spectra of 12 were assigned with the aid of distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple quantum coherence spectroscopy (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments. The positions of a (*E*)-caffeoyl group and a β -D-glucopyranosyl moiety were confirmed by HMBC spectroscopy (Figure 2). Namely, long-range correlations were observed between the following proton and carbon pairs: 1''-H and 3-C; 6''-H and 9''-C. Comparison of the ^{13}C -NMR data for tamarixetin 3-O- β -D-glucopyranoside [20] with those for 12 revealed an acylation shift around the 6'' position. From this evidence, the chemical structure of junsainoside A was determined to be tamarixetin 3-O-(6''-O-*E*-caffeoyl)- β -D-glucopyranoside (12).

Junsainoside A (12): A pale yellow powder, $[\alpha]_D^{21} -52.0^\circ$ ($c=0.1$, MeOH). HR-MALDI-TOF-MS Calcd for $C_{31}H_{28}O_{15}Na$ ($M+Na$) $^+$: 663.1320; Found: 663.1327, UV [MeOH, nm, ($\log \epsilon$): 255 (4.28), 329 (4.32). IR (KBr, cm^{-1}): 3400, 1689, 1655, 1605, 1514, and 1016. 1H -NMR (CD_3OD , 500 MHz) δ : 3.35 (1H, m, 4''-H), 3.43 (1H, m, 3''-H), 3.45 (1H, m, 5''-H), 3.50 (1H, m, 2''-H), 3.88 (3H, s, 4'-OCH $_3$), 4.28 (2H, m, 6''-H), 5.26 (1H, d, $J=7.3$ Hz, 1''-H), 6.07 (1H, d, $J=2.0$ Hz, 6-H), 6.12



(1H, d, $J=16.0$ Hz, 8'''-H), 6.25 (1H, d, $J=2.0$, 8-H), 6.79 (1H, d, $J=8.3$ Hz, 5'''-H), 6.80 (1H, d, $J=8.8$ Hz, 5'-H), 6.91 (1H, dd, $J=8.3$, 2.0 Hz, 6'''-H), 7.04 (1H, d, $J=2.0$ Hz, 2'''-H), 7.39 (1H, d, $J=16.0$ Hz, 7'''-H), 7.56 (1H, dd, $J=8.8$, 2.0 Hz, 6'-H), 7.57 (1H, d, $J=2.0$ Hz, 2'-H). ^{13}C -NMR (CD_3OD , 125 MHz) δ : 159.2 (C-2), 135.2 (C-3), 179.4 (C-4), 162.9 (C-5), 99.8 (C-6), 165.8 (C-7), 94.7 (C-8), 158.4 (C-9), 105.6 (C-10), 123.1 (C-1'), 123.4 (C-2'), 115.9 (C-3'), 149.8 (C-4'), 115.9 (C-5'), 123.4 (C-6'), 56.5 (4'-OCH $_3$), 103.8 (Glc-1''), 75.6 (Glc-2''), 78.0 (Glc-3''), 71.8 (Glc-4''), 75.9 (Glc-5''), 64.3 (Glc-6''), 127.6 (C-1'''), 146.8 (C-2'''), 149.3 (C-3'''), 150.5 (C-4'''), 116.4 (C-5'''), 124.2 (C-6'''), 146.8 (C-7'''), 115.0 (C-8'''), 168.8 (C-9'''). Positive-ion MALDI-TOF-MS: m/z 663 ($M+Na$) $^+$ (Figure 2).

Reagents

Human TNF- α quantkine ELISA kit, human total adiponectin/Acrp30 quantkine ELISA Kit and recombinant human adiponectin/Acrp30 were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant TNF- α was purchased from Life Technologies (MD, USA). Kaempferol 3-O-glucoside and tiliroside were purchased from Extrasynthese (Cedex, France). Radioimmunoprecipitation assay (RIPA) buffer, protease and phosphatase inhibitor cocktail, and Pierce Western Blotting Substrate Plus were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Goat anti-hyaluronan synthase (HAS) 2 IgG was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit anti-hyaluronan binding protein (HABP) 2 IgG was purchased from Abnova (Taipei, Taiwan). Mouse anti- β -actin (H-196) IgG and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, anti-goat IgG and anti-mouse IgG were purchased from Millipore (Billerica, MA, USA).

Cells

3T3-L1 (JCRB9014) and human diploid fibroblasts (TIG-108, JCRB0537) were obtained from the National Institute of Biomedical Innovation (Osaka, Japan). Normal human neonatal fibroblasts (NB1RGB, RCB0222) were obtained from Riken Bio Resources Center (Tsukuba, Japan). Human subcutaneous preadipocyte culture kit (PoieticsTM) was obtained from Lonza (Basel, Switzerland).

Lipid accumulation in cultured 3T3-L1 and human subcutaneous adipocytes

3T3-L1 cells (1×10^4 cells/200 μ L) suspended in Dulbecco's modified essential medium (D-MEM) containing 10% fetal calf serum (FCS) were seeded onto a 48-well culture plate and cultured for 24 hr. The medium was then changed to a new one containing 10% FCS, 5 μ g/mL insulin, 0.25 μ M dexamethasone and 0.5 mM isobutylmethyl xanthine and the cells were cultured for 2 days to induce differentiation. The medium was then changed to a new one containing 10% FCS, 5 μ g/mL insulin and test sample dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1%. The medium was changed to a new one every other day and the cells were cultured for a total of 6 days. The cells were stained with oil red O and observed under a microscope. The dye in cells was dissolved in isopropanol to measure the absorption at 540 nm. For the evaluation of cytotoxicities of the compounds from water shield, MTT assay was performed according to Denizot et al. [21], with modification. Briefly, cells (5×10^3 cells/100 μ L) were seeded in a 96-well culture plate and treated by the same procedure as described above. Then MTT reagent was added to each well and the plate was incubated for 2-4 hr. After washing the plate with phosphate buffered saline, pH 7.4 (PBS, Gibco[®], Life Technologies, CA), the

formed formazan dye was dissolved in 0.04M HCl/isopropanol solvent completely and the absorbance was measured at 570 nm.

Human subcutaneous preadipocytes (2×10^4 cells/200 μ L) suspended in the growth medium attached to the kit were seeded onto a 48-well culture plate and cultured for 24 hr. The medium was changed to the differentiation medium containing test sample (final concentration of DMSO: 0.1%) for 10 days. The medium was then collected and stored at -20°C . The contents of TNF- α and adiponectin were determined using a commercial kit. The cells were stained with oil red O and absorbance was measured as described above.

Fibroblast damage induced by TNF- α

NB1RGB cells (5×10^3 cells/100 μ L) suspended in D-MEM containing 10% FCS were seeded onto a 96-well culture plate and cultured for 24 hr. Then, sample (final concentration of DMSO: 0.1%) and TNF- α (10 ng/mL) were added and cultured for 20 hr. The cytotoxicity by TNF- α was evaluated by MTT assay as described above.

Evaluation of HAS2 and HAPB2 expression in fibroblasts induced by adiponectin

TIG-108 cells (1×10^5 cells/2 mL) suspended in D-MEM containing 10% FCS were seeded onto a 6-well culture plate and cultured for 24 hr. The medium was changed to D-MEM containing 0.5% FCS and cultured for 24 hr. Then, sample (final concentration of DMSO: 0.1%) and adiponectin (1 ng/mL) were added and cultured for 72 hr. Cells were collected with 200 μ L of RIPA buffer containing protease and phosphatase inhibitor cocktail. The protein concentration in the cell lysate was adjusted with RIPA buffer to 0.5 mg/mL. The lysate was mixed with the same volume of Laemmli sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 25% glycerol and 0.01% bromophenol blue) and heated at 95°C for 5 min. The sample solution (15 μ L) was electrophoresed on 10% SDS gel. Separated protein was then transferred to a polyvinylidene fluoride membrane. The membrane was treated with primary antibodies at the following dilution ratios: anti-HAS2 (1: 1,000), anti-HAPB2 (1: 2,000) and anti- β -actin (1: 10,000) for 1 hr. HRP-conjugated anti-goat IgG (1:25,000) or anti-rabbit IgG (1: 25,000) was used as the secondary antibody. Detection was performed using Pierce Western Blotting Substrate Plus and an imaging system (Image Quant LAS500, GE Health Care, Fairfield, CT, USA).

Statistics

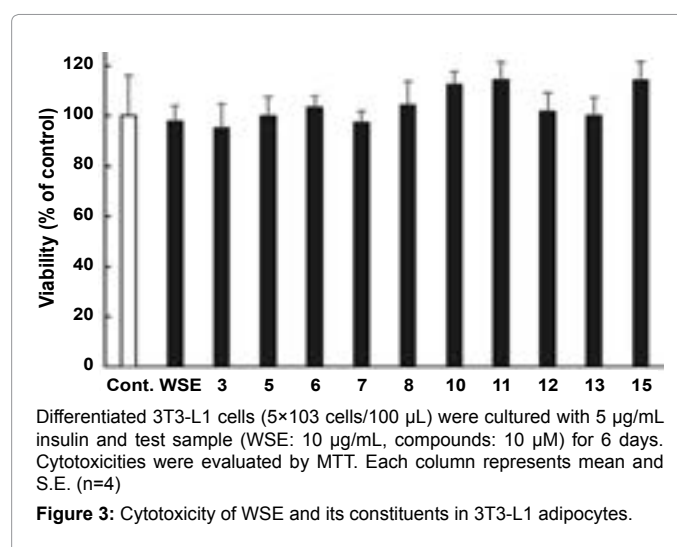
The results are expressed as means and S.E. Significance of the

differences was examined by one-way ANOVA followed by Dunnett's test. Differences of $p < 0.05$ were considered significant.

Results

Effects of WSE and the constituents of water shield on lipid accumulation in 3T3-L1 and human cultured subcutaneous adipocytes

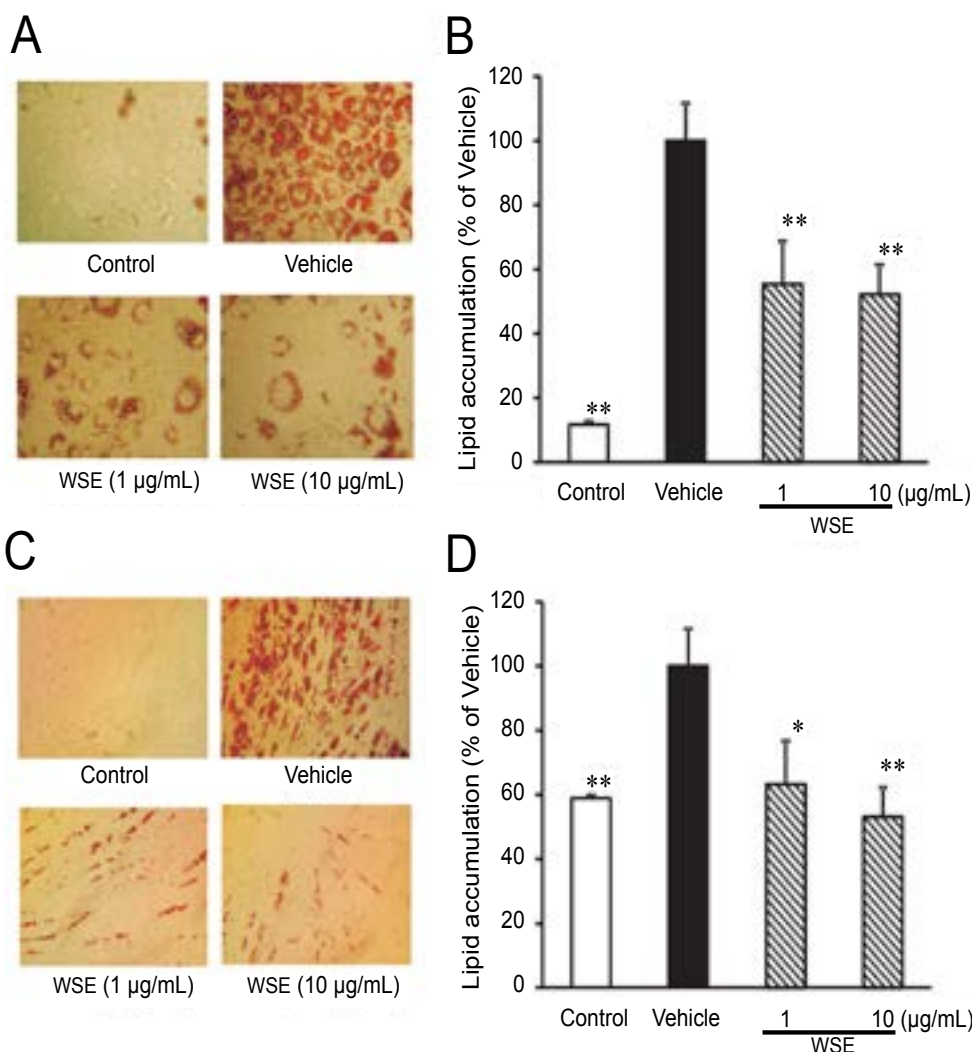
As shown in Figure 3, WSE and its constituents showed no cytotoxicity to 3T3-L1 adipocytes. Figures 4A and 4B illustrate lipid accumulation in 3T3L1 adipocytes. WSE (1, 10 μ g/mL) significantly suppressed lipid accumulation in the cells. WSE also suppressed lipid accumulation in human subcutaneous adipocytes (Figures 4C and 4D). We also examined the effect of the constituents of water shield extract on lipid accumulation in both cultured cells. Ethyl gallate (3), caffeoyl glucose (5), hypolaetin 7-O-glucoside (6), junsainoside A (12) and kaempferol (15) significantly suppressed lipid accumulation at 10 μ M in 3T3-L1 (Table 1). Ethyl gallate (3), 6 and 12 exhibited suppressive activity of more than 50% at 10 μ M. In human subcutaneous adipocytes, 3, ferulic acid (4), 5, 6, quercetin 3-O-(6"-galloyl) glucoside (8), quercetin 3-O-glucoside (10), junsainoside A (12) and kaempferol suppressed lipid accumulation at 1 μ M. Kaempferol 3-O-(6"-galloyl) glucoside (7) and gossypetin (11) significantly suppressed the accumulation at 10



	Inhibition (%)			
	3T3-L1		Human Subcutaneous Adipocytes	
	1 (μ M)	10	1 (μ M)	10
Ethyl gallate (3)	63.2 \pm 13.9*	58.9 \pm 14.8*	19.7 \pm 4.7**	34.9 \pm 1.9**
Ferulic acid (4)	–	–	23.3 \pm 3.1**	48.5 \pm 0.4**
Caffeoyl glucose (5)	42.3 \pm 15.3	43.6 \pm 13.4*	27.5 \pm 1.6**	35.9 \pm 3.1**
Hypolaetin 7-O-glucoside (6)	52.0 \pm 17.3*	55.8 \pm 14.1*	33.5 \pm 5.4**	42.5 \pm 1.6**
Kaempferol 3-O-(6"-galloyl) glucoside (7)	25.8 \pm 11.9	34.6 \pm 6.8	13.5 \pm 4.9	25.5 \pm 3.8**
Quercetin 3-O-(6"-galloyl) glucoside (8)	35.8 \pm 18.5	10.8 \pm 15.2	15.4 \pm 3.7*	21.3 \pm 4.5**
Quercetin 3-O-glucoside (10)	2.6 \pm 21.6	12.8 \pm 16.6	17.4 \pm 3.9*	22.0 \pm 2.6**
Gossypetin (11)	35.9 \pm 7.6	38.5 \pm 11.3	12.6 \pm 4.3	28.4 \pm 4.3**
Junsainoside A (12)	44.2 \pm 8.0**	52.8 \pm 9.0**	36.6 \pm 6.2**	45.5 \pm 2.4**
Quercetin (13)	30.7 \pm 17.7	31.1 \pm 1.5	–	–
Kaempferol (15)	35.6 \pm 7.5**	39.9 \pm 5.6**	31.4 \pm 4.4**	40.3 \pm 2.4**

Differentiated 3T3-L1 cells (1×10^4 cells/200 μ L) or subcutaneous preadipocytes (2×10^4 cells/200 μ L) were cultured with sample for 6 or 10 days. Accumulated lipid was evaluated by oil red O staining. Each value represents mean and the S.E. (n=4). Asterisks denote significant differences from the control group at *: $p < 0.05$, **: $p < 0.01$.

Table 1: Effects of the Constituents Isolated from Water Shield on Lipid Accumulation in Differentiated 3T3-L1 and Human Subcutaneous Adipocytes.



Differentiated 3T3-L1 cells (1×10^4 cells/200 μL) were cultured with 5 $\mu\text{g/mL}$ insulin and sample for 6 days. The cells were stained with oil red O. Each column represents mean and S.E. (n=4). Control and vehicle refer to differentiated and non-differentiated cells. Asterisks denote significant differences from the control group at *: $p < 0.05$, **: $p < 0.01$

Figure 4: Effects of WSE on lipid accumulation in 3T3-L1 cells (A, B) and human cultured subcutaneous adipocytes (C, D).

μM . Among the constituents, 4, 6, 12 and 15 exhibited a suppressive effect of more than 40% at 10 μM .

Effects of WSE and the constituents of water shield on TNF- α and adiponectin production from human cultured subcutaneous adipocytes

We evaluated the effect of WSE on TNF- α and adiponectin production from subcutaneous adipocytes. TNF- α production was not suppressed by the treatment of WSE or the constituents (Table 2). On the other hand, WSE did not enhance adiponectin production; however, ethyl gallate (3) and junsainoside A (12) significantly enhanced adiponectin production at 10 μM .

Effects of WSE and the constituents of water shield on fibroblast damage induced by TNF- α

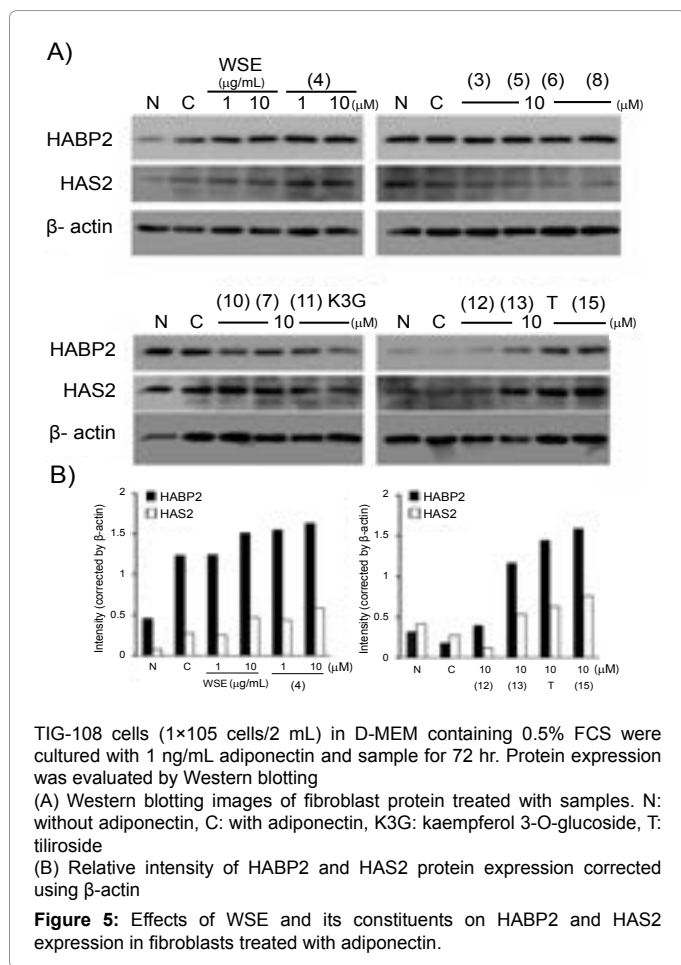
By the treatment of 10 ng/mL TNF- α , the formazan production indicating cell activity was reduced by approximately 41% compared

	Amount of adipocytokine production (% of control)			
	TNF- α		Adiponectin	
	1 ($\mu\text{g/mL}$)	10	1	10
WSE	110.6 \pm 2.2	93.2 \pm 3.0	118.2 \pm 8.9	111.7 \pm 5.1
	1 (μM)	10	1	10
Ethyl gallate (3)	89.9 \pm 5.34	86.7 \pm 4.5	122.2 \pm 5.8	138.3 \pm 10.0**
Ferulic acid (4)	97.7 \pm 3.1	83.8 \pm 6.1	120.6 \pm 8.5	123.3 \pm 7.6
Caffeoyl glucose (5)	112.0 \pm 10.4	103.6 \pm 2.4	105.7 \pm 5.2	107.6 \pm 3.7
Hypolaetin 7-O-glucoside (6)	116.5 \pm 6.1	109.3 \pm 4.7	120.6 \pm 11.3	104.1 \pm 4.1
Junsainoside A (12)	111.0 \pm 4.9	123.0 \pm 12.1	130.1 \pm 7.9	154.5 \pm 24.7*
Kaempferol (15)	97.0 \pm 3.3	92.7 \pm 3.2	–	–

Human subcutaneous preadipocytes (2×10^4 cells/200 μL) were differentiated and cultured with sample for 10 days. The contents of TNF- α and adiponectin were determined with an ELISA kit.

Each value represents mean with and S.E. (n=4). Asterisks denote significant differences from the control group at *: $p < 0.05$, **: $p < 0.01$.

Table 2: Effects of the Constituents Isolated from Water Shield on TNF- α and Adiponectin Production from Human Subcutaneous Adipocytes.



TIG-108 cells (1×10^5 cells/2 mL) in D-MEM containing 0.5% FCS were cultured with 1 ng/mL adiponectin and sample for 72 hr. Protein expression was evaluated by Western blotting

(A) Western blotting images of fibroblast protein treated with samples. N: without adiponectin, C: with adiponectin, K3G: kaempferol 3-O-glucoside, T: tiliroside

(B) Relative intensity of HABP2 and HAS2 protein expression corrected using β -actin

Figure 5: Effects of WSE and its constituents on HABP2 and HAS2 expression in fibroblasts treated with adiponectin.

	MTT assay (% of control)	
	1 (μ g/mL)	10
Normal	141.8 \pm 13.2*	
Control (with TNF- α)	100.0 \pm 8.2	
WSE	1 (μ g/mL)	161.6 \pm 11.3**
	1 (μ M)	10
Ethyl gallate (3)	129.8 \pm 6.0	135.1 \pm 4.6*
Ferulic acid (4)	104.9 \pm 8.1	84.3 \pm 5.4
Caffeoyl glucose (5)	112.6 \pm 3.3	129.1 \pm 8.6
Hypolaetin 7-O-glucoside (6)	120.4 \pm 14.2	98.2 \pm 6.2
Kaempferol 3-O-(6"-galloyl)-glucoside (7)	126.9 \pm 4.5	117.2 \pm 8.2
Quercetin 3-O-(6"-galloyl)-glucoside (8)	106.2 \pm 6.2	110.6 \pm 8.0
Quercetin 3-O-glucoside (10)	105.3 \pm 6.2	124.8 \pm 19.5
Gossypetin (11)	95.5 \pm 3.7	122.4 \pm 9.7
Junsainoside A (12)	97.8 \pm 2.7	92.3 \pm 7.7
Quercetin (13)	111.5 \pm 7.1	150.0 \pm 12.6*
Kaempferol (15)	74.4 \pm 3.0*	90.1 \pm 2.0

NB1RGB cells (5×10^3 cells/100 μ L) in D-MEM containing 10% FCS were cultured with sample and 10 mg/mL TNF- α for 20 hr. Cell damage was evaluated by MTT assay.

Each value represents mean and S.E. (n=6). An asterisk denotes significant difference from the control group at *: $p < 0.05$

Table 3: Effects of WSE and the Constituents Isolated from Water Shield on Fibroblast Damage Induced by TNF- α .

with that of cells without TNF- α . In WSE-treated cells, the cell activity was significantly recovered from cell damage at 1 and 10 μ g/mL.

Among the constituents of water shield, ethyl gallate (3) and quercetin (13) significantly recovered the cell activity at 10 μ M (Table 3).

Effects of WSE and the constituents of water shield on the expression of HABP2 and HAS2 in fibroblasts treated with adiponectin

In the fibroblasts cultured with adiponectin, WSE (10 μ g/mL) enhanced HABP2 and HAS2 expression (Figure 5). Among the constituents in water shield, ferulic acid (4), quercetin (12) and kaempferol (15) enhanced the expression of HABP2 and HAS2 at 10 μ M. Glycosides (3, 5, 6, 7, 8, 10, 12) and gossypetin (11) did not enhance the expression of HABP2 and HAS2. Kaempferol 3-O-glucoside (K3G) used for a reference compound of 10 also did not enhance HABP2 and HAS2 expression. On the other hand, tiliroside, a reference compound of 12, enhanced the expression of both.

Discussion

As a preliminary experiment using subcutaneous adipocytes, we examined the anti-adipogenic effect of test samples in differentiated 3T3-L1 adipocytes. As a result, WSE significantly suppressed lipid accumulation in adipocytes. Among the constituents of water shield, ethyl gallate (3), caffeoyl glucose (5), hypolaetin 7-O-glucoside (6), junsainoside A (12) and kaempferol (15) exhibited suppressive activity at 10 μ M. However, specific similarity in their moieties could not be found to exhibit anti-adipogenic activity. Then, the anti-adipogenic effect of WSE and its constituents was evaluated in subcutaneous adipocytes. WSE and its compounds (3, 5, 6, 12 and 15) suppressed lipid accumulation in differentiated subcutaneous adipocytes at a lower concentration than for 3T3-L1 adipocytes. Moreover, ferulic acid (4), kaempferol 3-O-(6"-galloyl) glucoside (7), quercetin 3-O-(6"-galloyl) glucoside (8), quercetin 3-O-glucoside (10) and gossypetin (11) exhibited anti-adipogenic activity at 10 μ M. These results suggest that WSE suppresses subcutaneous lipid accumulation effectively compared with that of abdominal adipose tissue and many polyphenols in water shield are involved in the activity.

To evaluate the direct effect of WSE on adipocytokine release, we measured TNF- α and adiponectin released from subcutaneous adipocytes. As WSE enhanced neither TNF- α nor adiponectin production, WSE was found not to affect to adipocytokine release directly. In general, adipocytokine production is changed toward lipid accumulation in adipocytes [22]. Thus, WSE is thought to attenuate adipocytokine release indirectly through the suppression of excessive lipid accumulation in adipocytes. However, high concentrations of 3 and 12 enhanced adiponectin release among selected compounds, showing relatively potent anti-adipogenic activity in subcutaneous adipocytes. Regarding naturally occurring compounds with similar chemical structures to 3 and 12, a green tea catechin with a galloyl moiety (epicatechin gallate) has been reported to increase serum adiponectin in hypertensive rats [23]. On the other hand, two compounds with somewhat similar structures to 12 have been reported to enhance adiponectin production. A citrus polymethoxy flavonoid, nobiletin, enhanced adiponectin secretion from ST-13 adipocytes [24]. Tiliroside, a glycosidic flavonoid with coumaric acid, also enhanced adiponectin production in obese mice [25]. The structure of tiliroside is quite similar to 12. From the activities of these known compounds, 3 and 12 may enhance adiponectin production when applied alone. Galloyl group, methoxyl group in flavonol and cinnamic acid moiety in flavonol glucoside may contribute to the adiponectin release from adipocytes. However, further studies are required to clarify the mechanism of these compounds.

Against the TNF- α -induced fibroblast damage, WSE, ethyl gallate (3) and quercetin (13) suppressed cell damage. TNF- α is released from not only inflammatory cells but also adipocytes. Besides inflamed skin, normal skin TNF- α level is thought to depend on recruitment from capillaries and adipose tissue, and increase with an increment of dermal adipose tissue. Hence, in addition to the anti-adipogenic effect, WSE and its compounds (3 and 13) are thought to be able to control skin TNF- α level.

In several skin disorders, including scleroderma [26], dermatomyositis [27] and psoriasis [28], dermal adiponectin expression has been reported to be lower than in normal skin. Adiponectin expression is also suppressed by morphological changes of the dermis related to abnormal and excessive lipid accumulation, such as cellulite [29] and type 2 diabetes [30]. Thus, adiponectin expression in dermis and subcutaneous adipose tissue is thought to play a crucial role in maintaining normal skin conditions. Upon the examination of hyaluronan binding protein and its synthase, co-treatment of adiponectin with WSE, ferulic acid (4), quercetin (13) and kaempferol (15) enhanced HAPB2 and HAS2 expression. As a preliminary examination, we treated fibroblasts with WSE alone. However, WSE did not enhance the expression of HAPB2 and HAS2 (data not shown). Thus, WSE and these compounds are thought probably to enhance hyaluronan production synergistically with adiponectin.

In conclusion, WSE and polyphenolic compounds in water shield were found to suppress lipid accumulation in subcutaneous adipocytes. Ethyl gallate and quercetin are involved in the suppressive effect of WSE on TNF- α -induced fibroblast damage. Moreover, ferulic acid, quercetin and kaempferol are found to enhance the expression of HAS2 and HAPB2 with the effect of adiponectin.

Conflict of Interest

This study was not supported by any other foundation except the Oryza Oil & Fat Chemical Co. Ltd.

References

- Kakuta M, Misaki A (2004) Structural characterization of water-shield mucilage of "Junsai (*Brasenia schreberi* J. F. Gmel)". *Foods Food Ingredients J Jpn* 209: 298-303.
- Legault J, Perron T, Mshvildadze V, Girard-Lalancette K, Perron S, et al. (2011) Antioxidant and anti-inflammatory activities of quercetin 7-O- β -D-glucopyranoside from the leaves of *Brasenia schreberi*. *J Med Food* 14: 1127-1134.
- Takahashi J, Toshima G, Matsumoto Y, Kimura F, Kiuchi T, et al. (2011) *In vitro* screening for antihyperlipidemic activities in foodstuffs by evaluating lipoprotein profiles secreted from human hepatoma cells. *J Nat Med* 65: 670-674.
- Drira R, Chen S, Sakamoto K (2011) Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3 T3-L1 cells. *Life Sci* 89: 708-716.
- Scazzocchio B, Vari R, Filesi C, D'Archivio M, Santangelo C, et al. (2011) Cyanidin-3-O- β -glucoside and protocatechuic acid exert insulin-like effects by upregulating PPAR γ activity in human omental adipocytes. *Diabetes* 60: 2234-2244.
- Wronska A, Kmiec Z (2012) Structural and biochemical characteristics of various white adipose tissue depots. *Acta Physiol (Oxf)* 205: 194-208.
- Bhattacharyya S, Dudeja PK, Tobacman JK (2010) Tumor necrosis factor alpha-induced inflammation is increased but apoptosis is inhibited by common food additive carrageenan. *J Biol Chem* 285: 39511-39522.
- Ezure T, Amano S (2007) Adiponectin and leptin up-regulate extracellular matrix production by dermal fibroblasts. *Biofactors* 31: 229-236.
- Rukachaisirikul T, Khamthong N, Sukpondma Y, Phongpaichit S, Hutadilok-Towatana N, et al. (2010) Cyclohexene, diketopiperazine, lactone and phenol derivatives from the sea fan-derived fungi *Nigrospora* sp. PSU-F11 and PSU-F12. *Arch Pharm Res* 33: 375-380.
- Takaoka S, Takaoka N, Minoshima Y, Huang JM, Kubo M, et al. (2010) Isolation, synthesis, and neurite outgrowth-promoting activity of illicinin A from the flowers of *Illicium anisatum*. *Tetrahedron* 66: 8354-8361.
- Galland S, Mora N, Abert-Vian M, Rakotomanomana N, Dangles O (2007) Chemical synthesis of hydroxycinnamic acid glucosides and evaluation of their ability to stabilize natural colors via anthocyanin copigmentation. *J Agric Food Chem* 55: 7573-7579.
- Siddiqui SA, Sen AB (1971) Hypolaetin 7-glucoside from *Juniperus macrospoda*. *Phytochemistry* 10: 434-435.
- Collins FW, Bohm BA, Wilkins CK (1975) Flavonol glycosides gallates from *Tellima grandiflora*. *Phytochemistry* 14: 1099-1102.
- Kadota S, Takamori Y, Khin N, Kikuchi T, Tanaka K, et al. (1990) Constituents of the leaves of *Woodfordia fruticosa* Kurz. I. Isolation, structure, and proton and carbon-13 nuclear magnetic resonance signal assignments of woodfruticodin (woodfordin C), an inhibitor of deoxyribonucleic acid topoisomerase II. *Chem Pharm Bull* 38: 2687-2697.
- Li M, Han X, Yu B (2003) Facile synthesis of flavonoid 7-O-glycosides. *J Org Chem* 68: 6842-6845.
- Markham KR, Ternal B, Stanley R, Geiger H, Mabry TJ (1978) Carbon-13 NMR studies of flavonoids-III: Naturally occurring flavonoid glycosides and their acylated derivatives. *Tetrahedron* 34: 1389-1397.
- Lee S, Park Y, Moon BH, Lee E, Hong S, et al. (2008) Substitution effect of hydroxyl groups on the 1H and 13C chemical shifts in hydroxyflavonols. *Bull Korean Chem Soc* 29: 1597-1600.
- Barakat HH, Souleman AM, Hussein SAM, Ibrahim OA, Nawwar MAM (1999) Flavonoid galloyl glucosides from the pods of *Acacia farnesiana*. *Phytochemistry* 51: 139-142.
- Tanaka T, Nakashima T, Ueda T, Tomii K, Kouno I (2007) Facile discrimination of aldose enantiomers by reversed-phase HPLC. *Chem Pharm Bull (Tokyo)* 55: 899-901.
- Fico G, Rodondi G, Flamini G, Passarella D, Tomé F (2007) Comparative phytochemical and morphological analyses of three Italian *Primula* species. *Phytochemistry* 68: 1683-1691.
- Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 89: 271-277.
- Shah A, Mehta N, Reilly MP (2008) Adipose inflammation, insulin resistance, and cardiovascular disease. *JPEN J Parenter Enteral Nutr* 32: 638-644.
- Potenza MA, Marasciulo FL, Tarquinio M, Tiravanti E, Colantuono G, et al. (2007) EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. *Am J Physiol Endocrinol Metab* 292: E1378-1387.
- Kunimasa K, Kuranuki S, Matsuura N, Iwasaki N, Ikeda M, et al. (2009) Identification of nobletin, a polymethoxyflavonoid, as an enhancer of adiponectin secretion. *Bioorg Med Chem Lett* 19: 2062-2064.
- Goto T, Teraminami A, Lee JY, Ohyama K, Funakoshi K, et al. (2012) Tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via activation of adiponectin signaling followed by enhancement of fatty acid oxidation in liver and skeletal muscle in obese-diabetic mice. *J Nutr Biochem* 23: 768-776.
- Arakawa H, Jinnin M, Muchemwa FC, Makino T, Kajihara I, et al. (2011) Adiponectin expression is decreased in the involved skin and sera of diffuse cutaneous scleroderma patients. *Exp Dermatol* 20: 764-766.
- Eimer MJ, Brickman WJ, Seshadri R, Ramsey-Goldman R, McPherson DD, et al. (2011) Clinical status and cardiovascular risk profile of adults with a history of juvenile dermatomyositis. *J Pediatr* 159: 795-801.
- Gerkowicz A, Pietrzak A, Szeptietowski JC, Radej S, Chodorowska G (2012) Biochemical markers of psoriasis as a metabolic disease. *Folia Histochem Cytobiol* 50: 155-170.
- Emanuele E, Minoretti P, Altabas K, Gaeta E, Altabas V (2011) Adiponectin expression in subcutaneous adipose tissue is reduced in women with cellulite. *Int J Dermatol* 50: 412-416.
- Statnick MA, Beavers LS, Conner LJ, Corominola H, Johnson D, et al. (2000) Decreased expression of apM1 in omental and subcutaneous adipose tissue of humans with type 2 diabetes. *Int J Exp Diabetes Res* 1: 81-88.

Citation: Shimoda H, Nakamura S, Hitoe S, Terazawa S, Tanaka J, et al. (2014) Anti-Adipogenic Polyphenols of Water Shield Suppress TNF- α -Induced Cell Damage and Enhance Expression of HAS2 and HAPB2 in Adiponectin-Treated Dermal Fibroblasts. *Nat Prod Chem Res* 2: 146. doi:10.4172/2329-6836.1000146