[Original Article]

Study of Inhibitory Effect of Polyphenols from *Fragaria ananassa* on Fat Accumulation in Rat White Adipocyte

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[ABSTRACT]

On the study of polyphenols from *Fragaria ananassa* fruit, we reported that some polyphenols showed inhibition of metabolic enzyme, cytochrome P450. Continuous study of health effects of *F. ananassa* fruit, we isolated a new quercetin glycoside, flagarin, quercetin-3-*O*- β -glucuronyl- (2 $\rightarrow$ 1)- β -D-xyloside along with ten known compounds. Those compounds showed inhibitory activity of fat accumulation in rat white adipocyte. Among the isolated compounds, strictinin and the new compound, flagarin showed high inhibitory activity of fat accumulation in rat white adipocyte.

Graphical abstract

We isolated a new quercetin glycoside, flagarin and ten known compounds from *Fragaria ananassa*. Those compounds showed fat accumulation inhibition in rat white adipocyte.

[Key words] *Fragaria ananassa*, flavonoid glycoside, fat accumulation inhibition, white adipocyte

[Abbreviations]

UV: Ultra Violet, NMR: Nuclear Magnetic Resonance, CD: Circular Dichroism, HPLC: High Performance Liquid Chromatography, ODS: Octadecylsilane, HRFABMS: High Resolution First Atom Bombardment Mass Spectrometry

INTRODUCTION

Obesity has become one of the social health problems. It increases the risk of chronic diseases such as “diabetes”, “hypertension”, “dyslipidemia”, “coronary artery disease” etc. Recently studies on the effects of dietary polyphenols in the prevention of obesity indicated that polyphenols reduce obesity through several mechanisms. Multiple mechanisms for anti-obesity imply the complexity of the biological functions and also the structural variation of polyphenols. Polyphenols have shown to have variable activity such as antioxidant¹⁾, antimicrobial²⁾, anti-allergy³⁾, and so on.

In the course of our program to study polyphenols from plant sources, we reported the isolation of some polyphenols from fruits of strawberry, *Fragaria ananassa* and inhibition of those on the metabolic activity of cytochrome P-450⁴⁾. Antioxidant activity of strawberry polyphenols such as ellagic acid and some flavonoids⁵⁾, antibacterial activity⁶⁾, inhibition of COX enzymes⁷⁾ and carcinogenic prevention⁸⁾ have been reported.

We report here the isolation of polyphenols which showed inhibitory activity of fat accumulation in rat white adipocyte, from the edible part of *F. ananassa*.

Along with ten known compounds, we isolated a new quercetin glycoside, flagarin, quercetin-3-*O*- β -

glucuronyl-(2 → 1)- β -D-xyloside and determined the structure by spectroscopic analysis and chemical degradation. Among the isolated compounds, strictinin and the new compound, flagarin showed high and concentration dependent inhibitory activity of fat accumulation in rat white adipocyte.

MATERIALS AND METHODS

General Experimental Procedures

UV spectra were obtained on a Shimadzu UV-1600 spectrophotometer. Optical rotations were measured on a Horiba SEPA-300 polarimeter. MS spectra were recorded on a JEOL JMS SX-102 mass spectrometer. ^1H , ^{13}C NMR spectra were measured on a JEOL ECA-600 spectrometer with deuterated solvents as internal standards. CD spectra were obtained on a JASCO J-820 spectrometer. Multi spectrophotometer was used Viento XS, Daiippon Sumitomo Pharma. Thermo ELECTRON HEPA CLASS 100 was used for cell incubation. The HPLC system consisted of a Waters 2487 Dual λ Absorbance Detector and a Waters 600 Pump. Columns used for separation were Develosil[®] ODS-HG-5; 20 x 250 mm (NOMURA CHEMICAL), Develosil[®] ODS-UG-5; 20x250 mm (NOMURA CHEMICAL), Develosil[®] Packed Column C30-UG-5; 20x250 mm (NOMURA CHEMICAL), Inertsil[®] NH2; 20x250 mm (GL Sciences). Column chromatography was performed on DIAION[®] HP-20 (Mitsubishi Chemical Corporation), Sephadex LH-20 (Pharmacia), or Wakogel[®] 40C₁₈ (Wako). Silica gel 60 F₂₅₄ (MERCK) and RP-18 F_{254s} (MERCK) were used for TLC. DPPH, SDS, Mildform[®] 20, and *p*-anisaldehyde were purchased from Wako Pure Chemical Industries. FBS, PC/ST, PBS, Rat white adipocyte, ascorbic acid, biotin, pantothenic acid, insulin, dexamethasone, 3-isobutyl-1-methylxanthine were purchased from TAKARA BIO. MTT reagent was purchased from Nacalai tesque. Trypsin/EDTA was purchased from GIBCO.

Plant Material

Edible part of *Fragaria ananassa* "tochi-otome" was provided from Biotherapy Development Research Center, Inc. *F. ananassa* "tochi-otome" used were cultivated in Tochigi prefecture, Japan.

Extraction and Isolation

Edible part of *F. ananassa* (8.00 kg) was extracted with hot water at 90 °C for 60 min. The crude extract (598 g) was separated on HP-20 chromatography (H₂O

→ 10% EtOH → 50% EtOH → EtOH) to yield four fractions. 50% EtOH fraction (30.6 g) having the activity of fat accumulation inhibitory was fractionated with LH-20 chromatography (10, 50 and 100% MeOH in H₂O) to get four fractions (Fr. A-D). Fr. B (932 mg) was chromatographed on ODS (15, 30, 40 and 100% EtOH in H₂O), and the fraction (125 mg) eluted with 15% EtOH was separated further by ODS chromatography (15, 30, 40 and 100% MeOH in 0.1% HCOOH). The fraction eluted with 30% MeOH in 0.1% HCOOH was purified by ODS HPLC (30% MeOH in 0.1% HCOOH) to isolate pelargonidin-3-*O*- β -glucoside (8, 7.36 mg). Fr. B (3.01 g) was chromatographed on ODS (10, 15, 20, 50 and 100% EtOH in H₂O) to get Fr. B-1 (98.0 mg, 10% aq EtOH) and Fr. B-2 (317.7 mg, 15-20% aq EtOH). Fr. B-1 (93.0 mg) was further chromatographed on ODS (H₂O; 5, 15, 20, 50 and 100% MeOH in 0.05% HCOOH). Fr. B-1-1 (34.76 mg, 15-25% MeOH in 0.05% HCOOH) was purified by ODS HPLC (20% MeOH in 0.1% HCOOH) to isolate 4-*O*- β -glucopyranosyl-*p*-coumaric acid (2, 7.36 mg) and (2*R*,3*R*)-2,3-dihydroquercetin-7-*O*- β -glucoside (7, 1.44 mg). Fr. B-1-2 (25.76 mg, 25% MeOH in 0.05% HCOOH) was purified by ODS HPLC (20% MeOH in 0.1% HCOOH) to isolate brevifolin carboxylic acid (4, 6.45 mg). Fr. B-2 (309.4 mg) was fractionated by ODS flash chromatography (H₂O; 15, 30, 40 and 100% MeOH in 0.05% HCOOH) to get Fr. B-2-1 (71.32 mg, 30% MeOH in 0.05% HCOOH), Fr. B-2-2 (54.61 mg, 30% MeOH in 0.05% HCOOH), and Fr. B-2-3 (28.88 mg, 30% MeOH in 0.05% HCOOH). Fr. B-2-1 (71.32 mg) was purified by ODS HPLC (17% CH₃CN in 0.1% HCOOH) to isolate *trans-p*-coumaric acid (1, 3.31 mg) and (-)-evofolin B (5, 1.86 mg). Fr. B-2-2 (54.61 mg) was purified by ODS HPLC (19% CH₃CN in 0.1% HCOOH) to isolate *trans*-ferulic acid (3, 2.03 mg). Fr. B-2-3 (28.88 mg) was purified by ODS HPLC (19% CH₃CN in 0.1% HCOOH) to isolate flagarin (quercetin-3-*O*- β -glucuronyl-(2 → 1)- β -D-xyloside, 11, 20.82 mg). The part of Fr. C (616.0 mg) was separated by ODS chromatography (10, 30, 50 and 100% MeOH in H₂O), and the fraction (310.1 mg) eluted with 30% aq EtOH was further separated by ODS chromatography (10, 15, 20, 25, 30, 35, 40, 50 and 100% MeOH in 0.05% AcOH). The fraction eluted with 20% MeOH in 0.05% AcOH was purified by ODS HPLC (20% aq CH₃CN) to isolate quercetin-3-*O*- β -glucuronide (10, 9.30 mg). Fr. C (203.0 mg) was chromatographed on ODS (10, 30, 5 and 100% MeOH in H₂O). Elution with 50% aq MeOH gave the fraction (7.4 mg) which was purified by ODS HPLC (50%

MeOH in 0.1% HCOOH) to isolate kaempferol-3-*O*- β -glucoside (**9**, 2.88 mg). Fr. C (2.84 g) was chromatographed on ODS (10, 30, 50 and 100% MeOH in H₂O). Elution with 30% aq MeOH gave the fraction (1.31 g) which was subjected to flash chromatography on ODS (10, 15, 20, 30 and 100% MeOH in 0.05% AcOH). The fraction (545.9 mg) eluted with 20-30% MeOH in 0.05% AcOH was further fractionated by ODS flash chromatography (10, 15, 20, 30 and 100% MeOH in 0.05% HCOOH). Elution with 10% MeOH in 0.05% AcOH gave the fraction (22.6 mg) which was further purified by ODS HPLC (20% MeOH in 0.1% HCOOH) to isolate strictinin (**6**, 7.36 mg). Flagarin (quercetin-3-*O*- β -glucuronyl-(2 $\rightarrow$ 1)- β -D-xyloside, **11**). UV (H₂O) $\lambda_{\max}$ (log ϵ) 382 (4.00), 316 (3.91), 272 (4.23), 198 (8.58); ¹H-NMR (CD₃OD) δ 3.17 (H-5'''a, dd, J =11.7, 12.4 Hz), 3.31 (H-2'', m), 3.33 (H-3'', m), 3.45 (H-4'', m), 3.62 (H-4''/5'', m), 3.64 (H-3'', t, J =8.6 Hz), 3.74 (H-2'', dd, J =7.9, 8.6 Hz), 3.88 (H-5'''b, dd, J =11.7, 12.7 Hz), 4.74 (H-1'', d, J =6.5 Hz), 5.57 (H-1'', d, J =7.9 Hz), 6.17 (H-6, d, J =1.7 Hz), 6.36 (H-8, d, J =1.7 Hz), 6.85 (H-5', d, J =8.6 Hz), 7.57 (H-6', dd, J =2.1, 8.6 Hz), 7.68 (H-2', d, J =2.1 Hz); ¹³C-NMR (CD₃OD) δ 66.5 (C-5'''), 71.0 (C-4'''), 73.2 (C-5''), 74.9 (C-2'''), 77.0 (C-3'''), 77.5 (C-3''), 78.0 (C-4''), 82.0 (C-2''), 94.5 (C-8), 99.7 (C-6), 100.9 (C-1''), 105.3 (C-1'''), 105.9 (C-10), 116.1 (C-5'), 117.5 (C-2'), 123.1 (C-1'), 123.3 (C-6'), 135.1 (C-3), 146.0 (C-3'), 149.7 (C-4'), 158.4 (C-10), 158.5 (C-2), 163.1 (C-5), 165.7 (C-7), 174.9 (C-6''), 179.5 (C-4); HRFABMS m/z : 609.1097 [M-H]⁻ (calcd for C₂₆H₂₆O₁₇ 609.1091 Δ 0.6 mmu).

Acid hydrolysis of flagarin (**11**)

A solution of flagarin (**11**) (5 mg) in 2 N HCl (2 mL) was heated at 100 °C for 2 h. The mixture was neutralized by addition of amberlite MB3 in water and evaporated. The crude residue was partitioned with EtOAc-H₂O. The water layer afforded glucuronic acid and arabinose, which were identified with authentic samples by TLC analysis. The water layer was directly analyzed by HPLC (75% aq CH₃CN in 0.1% HCOOH 3 mL/min; UV detection at 210 and 254 nm) on ODS-NH₂ column. The retention time of the peak at 39-46 min coincided with that of xylose. The optical rotation of isolated xylose in this acid hydrolysis was identical with D-(+)-xylose (Tokyo Kasei [α]_D +20.11° c 0.93 H₂O).

Cell line and culture

Rat white preadipocytes (White Adipocyte Culture

kit, TAKARA BIO INC) in growth medium [DMEM high glucose medium containing fetal Calf Serum (0.05 mL/mL), endothelial cell growth supplement (0.004 mL/mL), epidermal growth factor (10 ng/mL), hydrocortisone (1 μ g/mL) and heparin (90 μ g/mL)] were grown in 24-well culture plates (10,000 cells/well) at 37 °C under 5% CO₂ for 72 h. The cells were differentiated in the differentiation medium [growth medium containing d-biotin (8 μ g/mL), 3-isobutyl-1-methylxanthine (44 μ g/mL), dexamethasone (400 ng/mL), L-thyroxine (9 ng/mL), ciglitazone (3 μ g/mL) and insulin (0.5 μ g/mL)] for 48 h. Subsequently, the cells were maintained in growth medium containing 0.5 μ g/mL of insulin (900 μ L/well).

Inhibition of fat accumulation assay

Compound **1-11** were dissolved in DMSO following by dilution with the growth medium containing insulin (1%). Concentration of DMSO was 0.2%. Those sample solutions (each 100 μ L) were added to the adipocyte mixture and incubated for 72 h. After removing the culture medium, the cells were washed twice with PBS, fixed with 10% Mildform® at 4 °C for 2 h. Removing Mildform was followed by washing the cells with milli-Q water and staining with Oil-red-O (six parts 0.5% Oil-red-O dye in isopropanol and four parts milli-Q water) for 15 min. After rinsing with 100 μ L of isopropanol/milli-Q water (3:2) and then with 100 μ L of milli-Q water, lipid and Oil-red-O were dissolved in 100 μ L of isopropanol and quantified the lipid accumulation on a microplate spectrophotometer at 540 nm. The percentage of adiposity was calculated as follows: the percentage of adiposity (%) = [absorbance at 540 nm (sample)/absorbance at 540 nm (control)] $\times$ 100.

Measurement of cellular toxicity

In order to examine cellular toxicity, the decline in MTT reduction was measured. MTT was dissolved in PBS at the concentration of 5% w/v , and sterile filtered following to be stored at 4 °C. The cell count was adjusted to 1.0 $\times$ 10⁴ cells/mL. 1 mL of cell mixture was incubated for 72 h in 24-well plates at 37 °C.

The sample solution that was used for cytokine production assay was adjusted to 5, 10 and 50 μ M respectively using PBS and DMSO medium. LPS solution was also adjusted to 100 ng/mL using PBS medium. After incubation for 4 h, cells were treated with 20 μ L of each concentration of samples and 20 μ L of LPS solution for 24 and 48 h. As a control, cells were treated with 20 μ L of DMSO and 20 μ L of LPS solution. After

the stipulated time, 20 μ L of MTT solution was added to each well and incubated for 4 h under 5% CO₂ with light shielding. 100 μ L of SDS-DMF solution was added to each well and incubated for more 12 h. The color absorbance of each well was recorded at 570 nm in the medium with a reference serving as blank. The cell viability was calculated as follows: cell viability (%) = [absorbance at 570 nm (sample) / absorbance at 570 nm (control)] $\times$ 100.

Statistical Analysis

All values are presented as Mean $\pm$ SD. The data were analyzed by one-way analysis of variance (ANOVA). The differences compared with control group were assessed using Dunnett's multiple tests. Statistical significance was considered at $P < 0.05$.

RESULTS

Edible parts of *F. ananassa* were extracted with hot water at 90 $^{\circ}$ C to yield a crude extract. This crude extract was subjected to HP-20 column chromatography. The fraction eluted with 50% EtOH was separated by column chromatography over LH-20, ODS, and ODS on HPLC to isolate eleven compounds, *trans-p*-coumaric acid (1)⁹, 4-*O*- β -glucopyranosyl-*p*-coumaric acid (2)¹⁰, *trans*-ferulic acid (3)¹¹, brevifolin carboxylic acid (4)¹², (-)-evofolin B (5)¹³, strictinin (6)¹⁴, (2*R*, 3*R*)-2,3-dihydroquercetin-7-*O*- β -glucoside (7)^{15, 16}, pelargonidin-3-*O*- β -glucoside (8)¹⁷, kaempferol-3-*O*- β -glucoside (9) and quercetin-3-*O*- β -glucuronide (10)¹⁸, and a new flavonoid flagarin (11). The structures of polyphenols isolated in this study are shown in Fig.1.

Fig.1 Chemical structures of 1-11 isolated from strawberry

The HRFABMS for flagarin (**11**) showed a mass peak at m/z 609.1097 $[M-H]^-$, suggesting the molecular formula $C_{26}H_{26}O_{17}$, corresponding fourteen degrees of unsaturation. 1H -NMR spectra showed the two sets of aromatic groups. The five aromatic proton signals (δ 6.17, 6.36, 6.85, 7.57, and 7.68) were similar to those of compound **9** which has flavon structure. Two anomeric proton signals (δ 4.74, 5.57) indicated the presence of two saccharides. The presence of β -glucuronic acid was derived by the 1H - 1H COSY, HMQC, and HMBC spectra (Fig. 2 and 3). The presence of xylose was determined by the comparison of the 1H -NMR and optical rotation of the hydrolysate of flagarin (**11**) to those of authentic sample. The structure of flagarin (**11**) was thus determined by 1-D and 2-D NMR analysis as quercetin-3- O - β -glucuronyl-(2 $\rightarrow$ 1)- β -D-xyloside.

The white adipocyte store excess energy as triglycerides *in vivo*. In the culture test with rat white preadipocyte, fat is accumulated in the culture differentiation process of cells, and changes to white adipocyte causing obesity. Compounds **1-11** were added to those preadipocyte cells and the effect on fat accumulation was assessed by the Oil-red-O Stain method. Most of the isolated compounds showed significant inhibitory effect on fat accumulation in rat white adipocyte (Fig. 4 and 5).

DISCUSSION

Isolated compounds except for compound **3** and **8** showed inhibitory activity on fat accumulation in rat white adipocyte. In phenylpropanoid group (compound **1-3**), the presence of 3-OMe on phenyl is considered

Fig.2 Planar structure of flagarin (**11**)

to be responsible for the fat accumulation inhibitory activity. Among the compounds **2**, **4**, **6** and **11** showing fat accumulation inhibition, strictinin (**6**) and the new compound, flagarin (**11**) showed higher inhibitory activity of fat accumulation in rat white adipocyte.

To ensure that fat accumulation inhibitory activity is not due to cellular toxicity of the compounds, we examined the cytotoxicity of compounds **1-11** by MTT method. Experimental results (non-explicit) indicated that the compounds showed least cytotoxicity.

CONCLUSION

In this study, we isolated eleven polyphenols containing new diglucoside, flagarin (**11**) from edible part of *F. ananassa* hot water extract. This study indicated that strawberry contains various types of polyphenols which showed the inhibitory activity on fat accumulation in rat white adipocyte.

Whether the fat accumulation inhibitory activity to the white fat cell is related to the obesity prevention action of the human is a future problem. It could be interesting if the strawberry that many people like is relevant to the obesity prevention.

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Fig.3 1H and ^{13}C -NMR spectral data of flagarin (**11**) in CD_3OD

Fig.4 Effects of polyphenolic compounds on adipogenesis in cultured rat white adipocyte

Fig.5 Morphological changes in rat white adipocytes treated with some polyphenolic compounds (50 μM) isolated from strawberry. Cells were stained with Oil Red O after cultivation (×200).

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