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Anti-adipocyte differentiation activity and flavonoid content determination by HPLC/UV analysis of tree sprouts

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Abstract The i*n vitro* anti-obesity activity of 12 species of tree sprouts in differentiated 3T3-L1 cells and the mechanisms underlying their activity were evaluated. (+)-Catechin and quercetin concentrations in the sprouts were analyzed by HPLC/UV at 270 and 254 nm, respectively. *Euonymus alatus* (EAT) and *Fraxinus mandschuria* (FMS) extracts at doses of 50 and 100 μg/mL inhibited the accumulation of lipid droplets in differentiated 3T3-L1 cells. Moreover, EAT and FMS downregulated the expression of the CCAAT/enhancer-binding protein-α, adipogenesis-related proteins peroxisome proliferator-activated receptor-γ, and adipocyte P-2α in differentiated 3T3-L1 cells. Tree sprouts with an abundant flavonoid content exerted the highest anti-obesity activity. Concentrations of total flavonoids were the highest in FMS (24.281 mg/g DW) sprouts. These findings could be used to develop health-promoting functional foods or supplements derived from tree sprouts.

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Introduction

Obesity is caused by adipocytes, and adipogenesis is the differentiation of pre-adipocytes into mature adipocytes [1,2]. Abnormal adipogenesis is the unbalanced accumulation of lipids occurring through the increased metabolic formation of fat, leading to excessive adipocyte differentiation and occurrence of dysfunctional adipocytes formation [3,4]. These complex metabolic processes regulate the peroxisome proliferator-activated receptor-γ (PPAR-γ) expression, the CCAAT/enhancer-binding protein (C/EBP) transcription factor family, and adipocyte P2 (AP2), all of which are associated with adipocyte differentiation [5-7]. Flavonoids and phenolic compounds in plants inhibit adipocyte proliferation and increase differentiation at the cellular level [8,9]. Flavonoids and phenolic compounds in plants inhibit the proliferation of adipocytes and increase the differentiation of them at the cellular level. This inhibition of adipocyte differentiation may decrease adipose tissue mass or inhibit the activity of adipose signaling pathways that promote adipogenesis [10].

"Flavonoids" refers to a class of polyphenolic secondary metabolites found in plants. It is derived from *flavus*, a Latin word meaning yellow. The basic 15-carbon skeleton of flavonoids consists of two phenyl rings (A and B) and one heterocyclic ring (C) containing embedded oxygen [11]. Flavonoids have anti-inflammatory, anti-allergic [12], antioxidant [13], and antimicrobial [14] effects. The flavonoid catechin is a plant secondary metabolite that belongs to the flavan-3-ol group; (+)-catechin, found in cocoa [15], green tea, and vinegar [16], is an enantiomer of a (−)-catechin that has two chiral centers on carbons 2 and 3. Catechin exerts antioxidant [17], hepatoprotective [18], anti-inflammatory [19], and anti-hypertensive [20] effects. It also aids the prevention of UVB-induced skin damage [21], and possesses potential anti-

carcinogenic activity [22,23]. Quercetin is a flavonol that is abundant in red onions and kale, as well as in other fruits and vegetables. It inhibits oxidation [24], exerts anxiolytic [25] and anti-bacterial [26] effects, has potential anti-diabetic activity [27], and promotes bone formation [28].

Tree sprouts refer to the firstly or secondarily sprouted leaf and sometimes green twig totally. It is consumed as a gourmet food in Korea during spring and its popularity is increasing because of its taste and functionality to health. From the past, over fifty species of tree sprouts has been used in Korea. For example, "Dureop", the sprout from apical buds of *Aralia elata*, is the most popular tree sprout in Korea and its health benefits are well-known. The present study aimed to examine the *in vitro* anti-obesity activities of tree sprouts and to source out the one with the highest quantity of flavonoids.

Materials and Methods

Plant materials

Tree sprouts of *Actinidia arguta* (AAG), *A. polygama* (APG), *A. kolomikta* (AKM), *Aralia elata* (AET), *Eleutherococcus sessiliflorus* (ESF), *Kalopanax septemlobus* (KSL), *Staphylea bumalda* (SBD), *Securinega suffruticosa* (SST), *Cedrela sinensis* (CSS), *Euonymus alatus* (EAT), *Fraxinus mandshurica* (FMS), and *Lycium chinense* (LCS) were collected in Experimental Forest of National Institute of Forest Science, Hwasung, Korea at April to May, 2020 considering the growth condition of each species. A voucher specimen was deposited at Division of Special Forest Products, National Institute of Forest Science, Suwon, Korea.

Instruments and reagents

Flavonoids were analyzed by HPLC using a Flexar QUATERNARY Pump (PerkinElmer Life and Analytical Sciences Inc., Waltham, MA, USA), an auto-sampler, and a PDA LC UV detector (PerkinElmer). (+)-Catechin (99%) and quercetin (99%) were obtained from Natural Product Institute of Science and Technology Anseong, Korea (www.nist.re.kr). Their structures are shown in Fig. 1. HPLC-grade water and acetonitrile were purchased from J. T. Baker (Phillipsburg, PA, USA). Acetic acid (99.7%) and ethanol were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea).

Sample preparation and stock solutions

Powdered tree sprout samples (10 g each) were extracted with ethanol (200 mL) for 3 h under reflux conditions; this process was repeated three times. The extracts were then filtered and evaporated to obtain crude extracts weighing from 0.8-5.0 g (AAG, 0.9 g; APG, 2.3 g; AKM, 0.8 g; AET, 1.3 g; ESF, 1.8 g; KSL, 1.7 g; SBD, 1.2 g; SST, 2.3 g; CSS, 1.3 g; EAT, 2.0 g; FMS, 5.0 g and LCS, 2.9 g). The crude extracts (20 mg each) were sonicated in methanol for 20 min, and then filtered through a 0.45-μm polyvinylidene difluoride (PVDF) membrane. Standard stock solutions of (+)-catechin and quercetin (1 mg each) were prepared following the same aforementioned procedure.

Cell culture

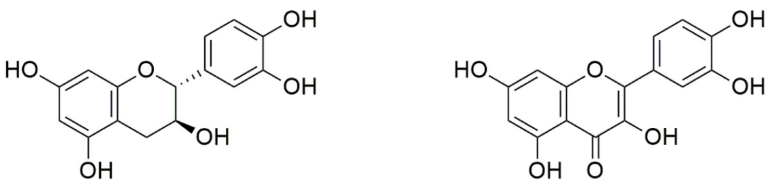
Pre-adipocytes (3T3-L1, CL-173^{TM}, ATCC Manassas, VA, USA) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% bovine calf serum (BCS, Merck KGaA, Darmstadt, Germany) and 100 μg/mL streptomycin and 100 U/mL penicillin (Thermo Fisher Scientific Inc.) at 37 °C under a humidified 5% CO_2 atmosphere.

Cell viability

Effects of the tree sprout extracts on cell viability were determined using CellTiter 96® AQueous One Solution Cell Proliferation Assays (Promega, Madison, WI, USA), as described by the manufacturer. The 3T3-L1 cells were seeded in 96-well plates for 24 h in complete medium and incubated with the extracts (at concentrations of 50 and 100 μg/mL) for 24 h and with the reagent for 2 h at 37 °C and under a 5% CO_2 atmosphere. Alamar blue reduction (from resazurin into resorufin) by viable cells was measured using a UV/visible spectrophotometer (Xma-3000PC, HumanCorp, Seoul, Korea) at 490 nm. The results are expressed as ratios (%) of viable to control cells [29]. Cell viability was assayed three times in triplicate.

Adipogenic differentiation

The 3T3-L1 pre-adipocytes were cultured in DMEM containing 10% BCS until they reached confluence (Day -2), and then maintained for 2 days (Day 0). Differentiation was induced using 10% fetal bovine serum (FBS) in DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, and



(+)-Catechin Quercetin

Fig. 1 Chemical structures of (+)-catechin and quercetin

5 μg/mL insulin. The medium was replaced 2 days later with DMEM containing 5 μg/mL insulin and 10% FBS. The medium was changed every 2 days for 8 days; then, the cells were collected for Oil Red O staining or western blotting [30].

Oil red O staining

Differentiated 3T3-L1 cells were washed twice with phosphate buffered saline, fixed in 4% paraformaldehyde for 1 h at room temperature, washed once with 60% isopropanol, and then stained with diluted oil red O working solution (0.42 g in 60% isopropanol) for 30 min. The stained cells were visualized and photographed using a CKX53 microscope (Olympus, Tokyo, Japan); then, the retained stain in 3T3-L1 cells was eluted with 100% isopropanol and the absorbance of these eluates was measured at 510 nm using an Xma-3000PC UV/visible spectrophotometer (Human Corporation).

Western blotting

Differentiated 3T3-L1 cells were lysed in a radio immuno precipitation assay buffer (Thermo Fisher Scientific Inc.) containing a protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA). Protein concentrations in the lysates were determined using the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). The proteins were resolved on 12% SDS-PAGE gels and blotted onto a PVDF membrane (Bio-Rad). Non-specific binding on the membrane was blocked with 5% bovine serum albumin (BSA; Biosesang, Seongnam, Korea) in Tris-buffered saline (TBS) containing 1% Tween 20 (TBS-T) for 1 h. The membranes were incubated with the primary monoclonal antibodies, i.e., anti-PPARγ (2443S), anti-C/EBPα (8178S), anti-AP2α (3215S) (all from Cell Signaling Technology, Boston, MA, USA) and anti-GAPDH antibodies (6C5, ab8245; Abcam Plc., Cambridge, UK) in 5% BSA overnight at 4 °C. The membranes were washed with TBS-T, and then incubated with 1:5000-diluted secondary anti-rabbit IgG-HRP (18-8816-33) and anti-mouse IgG-HRP (18-8817-33) monoclonal antibodies (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) for 1 h. The immunoreactive bands were visualized using an ECL western blotting substrate (Bio-Rad) and a ChemiDoc imaging system (Bio-Rad) and analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

HPLC/UV conditions

Flavonoids were quantified by an HPLC using a reverse-phase YMC-Pack Pro C18 column (4.6×250 mm, 5 μm) maintained at 25 °C. The injection volume was 10 μL and the flow rate was 1 mL/min. The mobile phase was 0.5% acetic acid in water (A) and acetonitrile (B). (+)-Catechin and quercetin were eluted in a gradient system as follows: 0 min, 95% A; 25 min, 80% A; 30 min, 60% A; 35 min, 100% B; 40 min, 100 B; 45 min, 95% A; and 55 min, 95% A; then (+)-catechin and quercetin were detected using the UV detector of the HPLC system at 270 and 254 nm, respectively. All samples were injected in triplicate.

Calibration curves

Working solutions to construct the calibration curves were prepared by serially diluting 1 mg/mL of the stock solutions of the tested compounds. The calibration functions of the flavonoids were calculated from the peak area (Y) and concentration (X, mg/mL), and the values are presented as means ± standard deviations (SD) (n =3).

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) and are presented as the means ± SD. Data were analyzed using one-way analysis of variance, and the means from multiple groups were compared using *Tukey post-hoc* tests.

Results and Discussion

Obesity is induced by excessive lipid storage which can lead to hyperlipidemia, fatty liver, type II diabetes, and cancer [31-33]. It is caused by the accumulation of intracellular triglycerides (TGs) *via* preadipocyte differentiation [1,2]. Previous investigations have studied the anti-obesity effects of natural products that exert these effects via the inhibition of excessive TG storage [34,35]. The current investigation focused on the anti-obesity activity and molecular mechanisms underlying the effects of tree sprout extracts, and their effects on 3T3-L1 cell viability *in vitro*. All the extracts of tree sprouts were not significantly cytotoxic at concentrations of 50 and 100 μg/mL, compared with untreated 3T3-L1 cells (Fig. 2). Therefore, we further investigated the anti-obesity activities of the extracts at these concentrations.

Adipogenesis is the differentiation of pre-adipocytes into adipocytes, during which lipid droplets accumulate within cells [3,4], which is a characteristic of obesity. Therefore, inhibiting adipogenesis can suppress obesity [2]. In the present study (Fig. 3), we assessed the effects of 12 tree sprouts extracts on the lipid accumulation (particularly, TGs) in differentiated 3T3-L1 cells by staining the lipid droplets within these cells with oil red O [36]. Incubating 3T3-L1 cells with 50 μg/mL of AAG, APG, AKM, AET, ESF, KSL, EAT, FMS, and LCS sprout extracts significantly decreased the number of lipid droplets, compared with the untreated control cells, and at a concentration of 100 μg/mL, AAG, APG, AKM, AET, ESF, EAT, and FMS extracts inhibited lipid droplet accumulation, compared with control cells. Furthermore, 50 and 100 μg/mL of the EAT and FMS extracts inhibited lipid droplet accumulation more effectively than the other extracts.

The molecular mechanisms underlying adipogenesis in 3T3-L1 cells involve several related factors [37]. The expression of differentiation-related transcription factors, such as PPAR-γ, and C/EBP-α, -β, initiates adipogenesis when pre-adipocytes are stimulated by adipogenic mediators [38,39]. The PPAR-γ and C/

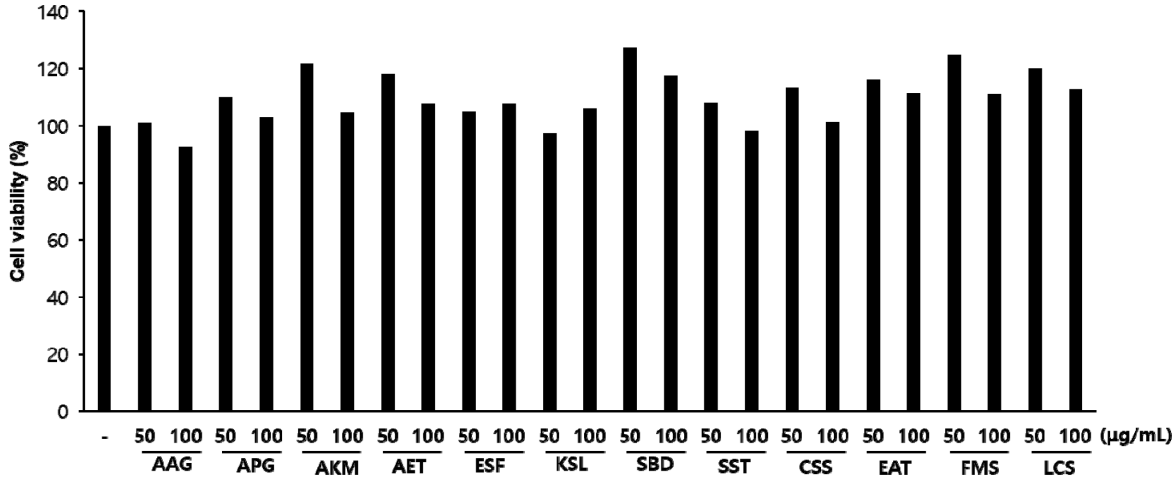


Fig. 2 Effects of tree sprout extracts on cell viability in 3T3-L1 cells

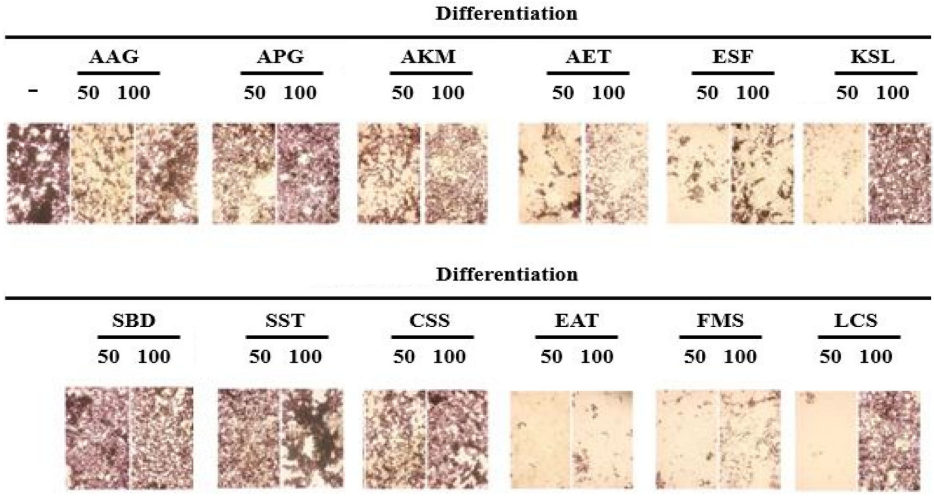


Fig. 3 Effects of tree sprout extracts on lipid droplet accumulation in differentiated 3T3-L1 cells

EBP-α activation upregulates the expression of target genes responsible for the adipocyte phenotype and lipid accumulation [39,40]. Adipogenesis also increases the expression of adipocyte-specific genes, including AP2, which induces fat accumulation and plays a significant role in lipogenesis [38]. We studied the molecular mechanisms underlying the anti-obesity activity of EAT and FMS by measuring the expression levels of the adipogenesis-related proteins PPAR-γ, C/EBP-α, and AP-2α in differentiated 3T3-L1 cells. We found that the genes encoding PPAR-γ, C/EBP-α, and AP-2α were downregulated in cells incubated with EAT and FMS sprout extracts, compared with the untreated cells (Fig. 4). EAT extract dose-dependently decreased the PPAR-γ, C/EBP-α, and AP-2α expression levels in a dose-dependent manner, whereas the FMS extracts dose-dependently decreased the PPAR-γ and C/EBP-α levels in differentiated 3T3-L1 cells in a dose-dependent manner. In particular, 50 and 100 μg/mL of EAT

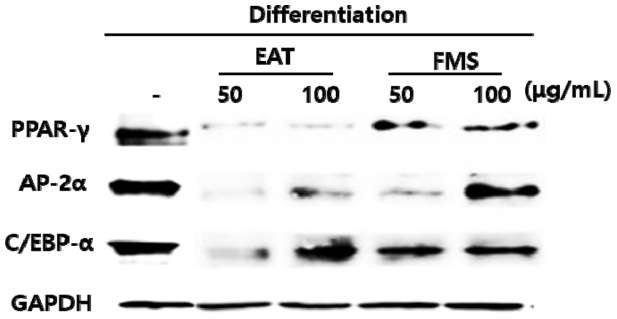


Fig. 4 Effects of tree sprout extracts on the protein levels of PPAR-γ, AP-2α, and C/EBP-α in differentiated 3T3-L1 cells. GAPDH was used as the loading control

extracts resulted in a greater decrease in PPAR-γ and AP-2α protein expression. Therefore, EAT and FMS extracts appear to

inhibit lipid accumulation by regulating the adipogenesis-related genes PPAR-γ, C/EBP-α, and AP-2α expression.

The retention times of (+)-catechin and quercetin upon HPLC separation were 18.5 and 35.8 min, respectively (Fig. 5), and their standard calibration curves showed the equations Y =4766.3X + 40318 and Y =42621X−2754, respectively. The calibration curves of flavonoids were constructed from the plots of peak areas relative to the concentrations of the corresponding standards using

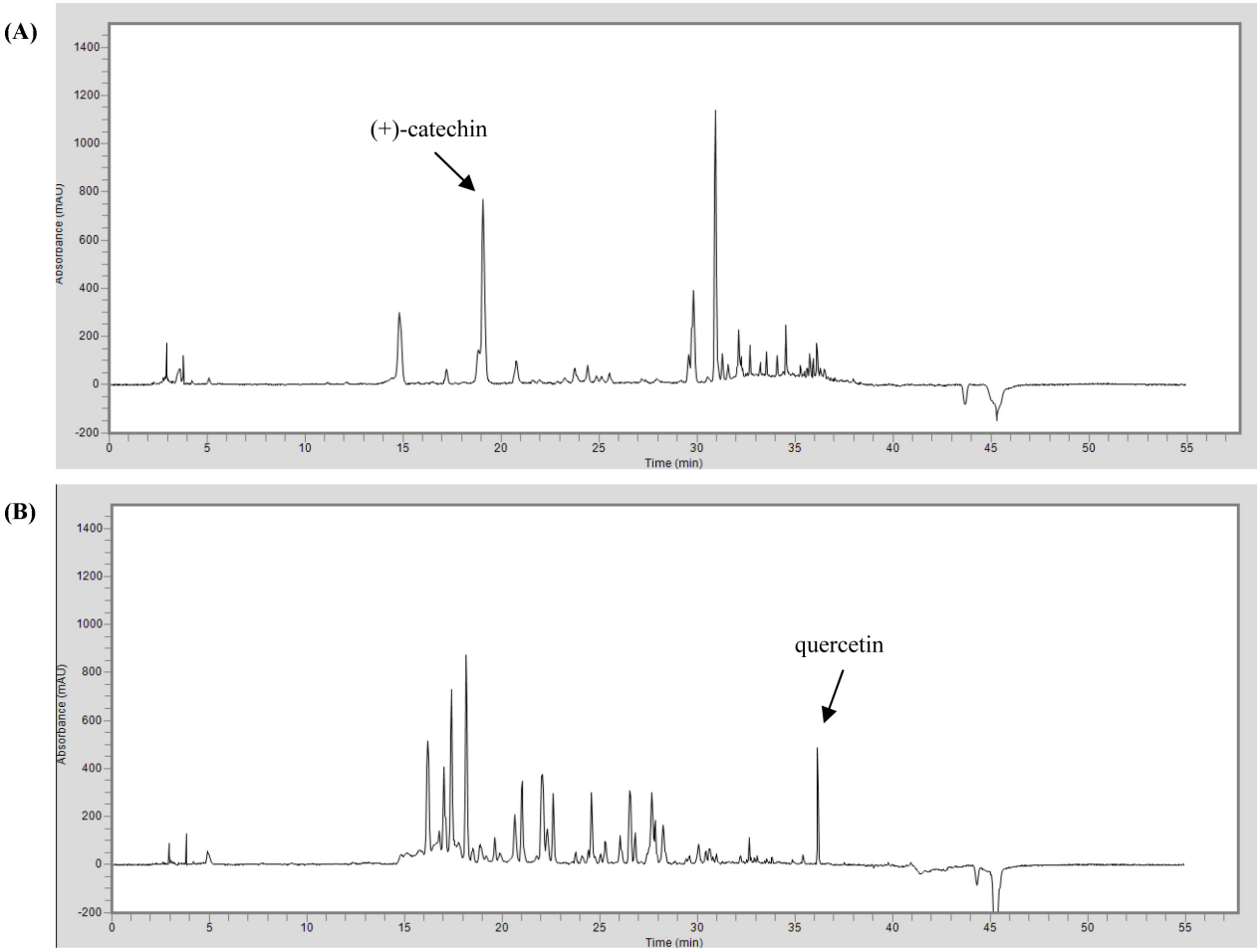


Fig. 5 High-performance liquid chromatography analyses of the ethanol extracts of FMS (A) and EAT (B) containing (+)-catechin and quercetin

Table 1 The contents of (+)-catechin and quercetin in tree sprouts

Sample	Content (mg/g DW)		
	(+)-Catechin	Quercetin	Total
AAG	0.229±0.031	0.006±0.000	0.235
APG	0.699±0.018	nd	0.699
AKM	0.071±0.071	0.006±0.001	0.077
AET	8.681±0.121	tr	8.681
ESF	3.894±0.779	tr	3.894
KSL	7.850±0.340	0.014±0.002	7.864
SBD	0.145±0.009	0.017±0.001	0.162
SST	0.208±0.077	0.012±0.004	0.220
CSS	0.757±0.173	0.059±0.004	0.816
EAT	0.418±0.010	0.189±0.004	0.607
FMS	24.186±0.565	0.095±0.004	24.281
LCS	4.559±0.650	nd	4.559

nd, not detected; tr, trace

linear regression analysis. The regression coefficients (r^2) for (+)-catechin and quercetin were 0.9998 and 0.9996, respectively. Peaks of (+)-catechin and quercetin were identified by HPLC in the extracts of the 12 tree sprouts. The contents of these compounds in the extracts were calculated (Table 1). More (+)-catechin than quercetin was significantly found in all extracts (Fig. 5), however FMS contained the highest levels of flavonoids.

This study showed that EAT and FMS most effectively inhibited lipid accumulation by regulating adipogenesis-related factors in 3T3-L1 cells. Furthermore, FMS contained a higher abundance of flavonoids. Therefore, we considered that tree sprouts containing high contents of flavonoids have anti-obesity activity. These results provide foundational information that may facilitate the development of health-promoting functional foods or nutritional supplements.

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