Vitexin, Orientin and Other Flavonoids from Spirodela polyrhiza Inhibit Adipogenesis in 3T3-L1 Cells

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To investigate the adipogenesis inhibitory effect on lipid accumulation, 3T3-L1 cells were treated with fractions and isolated flavonoids of Spirodela polyrhiza. An ethanol extract of S. polyrhiza was fractionated into three fractions. The butanol soluble fraction (SPB) exhibited potent antiadipogenesis activity and decreased C/EBPα and PPARγ protein expression level in 3T3-L1 cells without significant cytotoxicity. The flavonoids were isolated from SPB and their chemical structures were identified as chrysoeriol (1), apigenin (2), luteolin (3), vitexin (4), cosmosin (5), orientin (6) and luteolin-7-O-β-D-glucoside (7) by spectroscopic analysis. Studies on the adipogenesis and intracellular triglyceride accumulation inhibitory effect showed that compounds 4 and 6 had the highest activity and decreased C/EBPα and PPARγ protein expression level in 3T3-L1 cells. These results suggest that the flavonoids isolated from SPB, especially compounds 4 and 6, contribute to the inhibitory activity of S. polyrhiza in 3T3-L1 cells. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: vitexin; orientin; Spirodela polyrhiza; flavonoids; 3T3-L1; adipogenesis.

INTRODUCTION

Obesity is a state of accumulated energy that can cause additional metabolic diseases such as fatty liver, hyperlipidemia, hypertension, atherosclerosis, type II diabetes mellitus and cancer (Bray, 2000; Greenway and Smith, 2000). Recently, increases in obesity have been observed not only in developed countries but also developing countries (WHO, 2000). Despite some of their valued approaches for weight loss, drugs and food supplements have been reported to have serious side effects such as vomiting, headache, stomachache, and heart attack (Park et al., 2007b), so researchers have tried to identify sources that are more effective and have fewer side effects. Natural products, including traditional herbal remedies, have been reported as a subject for an obesity study in 3T3-L1 cells. Previously, the effect of ginsenosides, the major active components of panax ginseng, on lipid accumulation in 3T3-L1 adipocytes was investigated (Lee et al., 2009). It has also been reported that genistein, a soy-derived isoflavone, inhibits 3T3-L1 adipocyte differentiation (Zhang et al., 2009). 3T3-L1 cells are an appropriate preadipocyte in vitro model for studying differentiation and obesity metabolism (MacDougald and Lane, 1995), because adipocyte differentiation plays an important role during the induction of obesity (Shimomura et al., 1998). The causes of obesity are multifactorial. One of the causes is unregulated genes, such as the CCAAT-enhancer-binding proteins (C/EBPα), and peroxisome proliferator activated receptor γ (PPARγ) which stimulates lipid metabolism (Hindle et al., 2009).

Spirodela polyrhiza (L.) Schleid is an aquatic perennial plant distributed in most regions of Korea, Japan and China and has long been used in Oriental medicine in these countries for treating inflammation, urticaria and skin disease (Shin et al., 1982). The crude ethanol extract of a Spirodela species has been reported to have an inhibitory effect on preadipocyte proliferation (Cho et al., 2008). Nevertheless, the active compounds have not been clarified. Thus, the inhibitory effects of a butanol soluble fraction (SPB) and isolated compounds from SPB on adipogenesis of 3T3-L1 cells were investigated.

MATERIALS AND METHODS

Reagents. Dulbecco’s-modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Co. (Grand Island, NY, USA). Calf serum (CS) was purchased from HyClone (Logan, UT, USA) and dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin (INS) and Oil Red-O were obtained from Sigma (St Louis, MO, USA). Metformin (1,1-dimethylbiguanide, MP Bio Medicals, LLC, Solon, OH) used as a positive control (Kanda et al., 2008).
Plant material. *Spirodela polyrhiza* was purchased from a local herbal medicinal market in Korea (October 2008) and identified by one of the authors (Prof. KiHwan Bae). A voucher specimen (CNU-2683) has been deposited in the herbarium at the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and isolation. The air-dried whole *S. polyrhiza* plant (1.6 kg) was extracted with ethanol three times, each for 3 days at room temperature. The extract was concentrated *in vacuo* and then suspended in distilled water. The aqueous residue was partitioned and concentrated successively with hexane, methylene chloride and butanol to obtain a hexane soluble fraction (SPH, 75 g), methylene chloride soluble fraction (SPM, 42 g) and a butanol soluble fraction (SPB, 130 g). SPB, which exhibits potent nontoxic antiadipogenesis activity in 3T3-L1 cells, was subjected to silica gel column chromatography and eluted with a CHCl3:MeOH gradient (100:1 to 1:1). Thin layer chromatography (TLC) resulted in seven (B1–B7) fractions (Fr.). Fr. B1 was further chromatographed on a Sephadex LH-20 column using MeOH:H2O (3:1) and purified by preparative liquid chromatography (HPLC) with MeOH:H2O (60:40) to afford 1 (4.5 mg). Fr. B3 was further chromatographed on a Sephadex LH-20 column using MeOH:H2O (3:1) to yield 2 (15 mg). Fr. B5 was rechromatographed on a Sephadex LH-20 column using MeOH:H2O (3:1) and gave 3 (25 mg). Fr. B6 was rechromatographed on a Sephadex LH-20 column using MeOH:H2O (3:1) and purified by preparative HPLC with MeOH:H2O (60:40) as an eluent to afford 4 (6.5 mg) and 5 (7 mg). Fr. B7 was chromatographed on a Sephadex LH-20 column using MeOH:H2O (3:1) and purified by preparative HPLC with MeOH:H2O (45:65) as the eluent to yield 6 (9 mg) and 7 (8 mg). For the isolation, analytical TLC was performed on pre-coated Merck Fsc silica gel plates and visualized by spraying with anisaldehyde–H2SO4. Column chromatography was conducted using silica gel (Kiesel gel 70–230 mesh and 230–400 mesh, Merck, Whitehouse Station, NJ, USA) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). An HPLC system equipped with 321 pumps, a UV-visible 151 detector, GX-271 liquid handler with TRI-GENE automation system and 250 m columns was used. The isolated compounds (1–7) were identified by 1H and 13C nuclear magnetic resonance (NMR) spectroscopy. 1H (400 MHz) and 13C (100 MHz) NMR spectra were recorded on a Bruker DRX-400 with TMS as the internal standard.

Cell culture and adipocyte differentiation assay. The 3T3-L1 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in 5% CO2 at 37°C in DMEM containing 10% FCS and 1% penicillin–streptomycin. The cells were seeded in 96-well plates and incubated for 24 h at 5% CO2 and 37°C and then incubated in serum-free DMEM with samples for 24 h. The extent of MTT reduction to formazan within cells was quantified by measuring the absorbance at 490 nm using a microplate reader.

MTT viability assay. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described previously (Mosmann, 1983). The cells were seeded in 96-well plates and incubated for 24 h at 1% CO2 and 37°C and then incubated in serum-free DMEM with samples for 24 h. The extent of MTT reduction to formazan within cells was quantified by measuring the absorbance at 490 nm using a microplate reader.

Western blot analysis. 3T3-L1 adipocytes were harvested 9 days after the initiation of differentiation. The cells were incubated with the SPB or flavonoids at 2 days post-confluent at 37°C in a humidified 5% CO2 incubator. The cells were collected and lysed in lysis buffer (1% Triton X-100 in PBS). The total triglyceride content in cells was determined using a commercial triglyceride assay kit (TG-S reaction kit, Asan Pharm. Co., Seoul, Korea). The protein quantity was estimated with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The triglyceride content was expressed as the percent vs control (100%).

Statistical analysis. Experimental values are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). Data were analysed with a one-way ANOVA. A value of *p* < 0.05 was considered statistically significant.
RESULTS

Adipogenesis inhibition and western blotting of *S. polyrhiza*

The SPH, SPM and SPB were obtained from the *S. polyrhiza* ethanol extract. The cytotoxicity and adipocyte differentiation inhibitory activity of these fractions were evaluated by the MTT cell viability assay and adipocyte differentiation assay in 3T3-L1 cells. The results showed that SPH and SPM induced cytotoxicity of 3T3-L1 cells at concentrations of 20–200 μg/mL. Due to the cytotoxicity of SPH and SPM on 3T3-L1 cells, the adipocyte differentiation assay data were of no use for evaluating antiadipogenesis activity (Figs 1–3). SPB was the only fraction that showed significant 3T3-L1 adipocyte differentiation ability without cell toxicity (Fig. 1-1). The 3T3-L1 cells were treated with 20, 40, 100 and 200 μg/mL during differentiation and stained. As shown in Fig. 1-2, SPB decreased triglyceride accumulation in a dose dependent manner at concentrations of 20, 40, 100 and 200 μg/mL with no cytotoxicity. To identify related SPB adipogenesis mechanisms in 3T3-L1 cells, the C/EBPα and PPARγ protein expression level were analysed using western blot at concentrations of 40, 100 and 200 μg/mL SPB (Fig. 2). The results showed that SPB inhibited the expression of C/EBPα and PPARγ. C/EBPα and PPARγ are important major transcription factors, known as a key station protein in the adipocyte differentiation of 3T3-L1 cells (Tontonoz *et al.*, 1994).

Identified flavonoids from SPB and adipogenesis inhibition and western blotting

A phytochemical study was performed on SPB to identify the active nature of SPB, and seven compounds were isolated. Compounds 1–7 were identified as chrysoeriol (1) (Park *et al.*, 2007a), apigenin (2) (Lamyaa *et al.*, 2007), luteolin (3) (Geiger *et al.*, 1995), vitexin (4) (Omayma *et al.*, 2008), cosmosin (5) (Agrawal, 1989), orientin (6) (Jung *et al.*, 1999) and luteolin-7-O-β-d-glucoside (7) (Jin *et al.*, 2009) by spectroscopic methods and a comparison with the literature. The chemical structures of these identified compounds are shown in Fig. 3. Chrysoeriol has not been reported previously in *Spirodela* species. The effect of the isolated compounds on adipogenesis in 3T3-L1 cells was measured by Oil Red-O staining and cellular triglyceride accumulation was determined after differentiation. To determine non-toxic concentrations, 3T3-L1 cells were treated with various concentrations (25, 50 and 100 μM) of compounds 1–7, and the cell viability was measured. None

1) [Image]

2) [Image]

3) [Image]
of the compounds were cytotoxic at concentrations up to 100 μm, compared with the non-treated control \((p < 0.01)\). Chrysoeriol, apigenin, cosmosin and luteolin-7-O-β-D-glucoside also decreased lipid droplet accumulation by 23.1%, 22.9%, 17.6% and 22.7% at 100 μm, respectively \((p < 0.01)\). Vitexin and orientin decreased C/EBPα and PPARγ protein expression level in 3T3-L1 cells (Fig. 4B). The effects of the flavonoids on the inhibition of triglyceride accumulation in 3T3-L1 adipocytes are shown in Fig. 4D. The results demonstrated that the flavonoids caused an inhibition of triglyceride accumulation in the 3T3-L1 adipocytes. Vitexin and orientin revealed the highest inhibition of triglyceride accumulation (39.4% and 33.0%, at 100 μm, respectively) among seven flavonoids being tested.

**DISCUSSION**

In this study, three different fractions were obtained from the *S. polyrhiza* extract. The results showed that SPB had strong adipogenesis inhibitory activity without significant cytotoxicity. Seven flavonoids were isolated from the SPB and showed different activity. Further studies will be needed to determine the mechanism and the relation of the chemical structure of the flavonoids and the activity. Interestingly, some flavonoids such as quercetin, kaempferol, isorhamnetin have also been reported as having antiadipogenesis activity (Iwashita et al., 2001).

In conclusion, adipogenesis inhibitory effect of SPB and various flavonoids isolated from the SPB of *S. polyrhiza* have been elucidated as indicated by the decrease in triglyceride content. The SPB, vitexin and orientin inhibited adipogenesis of 3T3-L1 cells by decreasing expression of adipogenic transcription factors (C/EBPα and PPARγ).

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This study showed that flavonoids from SPB contributed to the inhibitory activity of SPB. However, the equally potent effect of the crude butanol fractions suggests that other compounds may also have an inhibitory effect on adipogenesis of SPB.

These results indicate that the flavonoids and other as yet unidentified compounds of *S. polyrhiza* may be useful for further investigations into their possible role for preventing obesity.

**Conflict of Interest**

The authors have declared that there is no conflict of interest.

**REFERENCES**


