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Original article

Quantification of the constituents of the traditional Korea medicine, Samryeongbaekchul-san, and assessment of its antiadipogenic effect

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ABSTRACT

Samryeongbaekchul-san (SBS) is a traditional herbal formula, which is used for the treatment of dyspepsia, chronic gastritis, and anorexia in Korea. To evaluate the quality of SBS decoction by quantifying its main constituents simultaneously using high-performance liquid chromatography coupled with photodiode array (HPLC–PDA) detection, and secondly to determine the antiadipogenic effect of SBS decoction. The main constituents in a 10-μL injection volume of the decoction were separated on Gemini C_{18} and Luna NH_2 columns (both 250 mm × 4.6 mm, 5 μm) at 40 °C using a gradient of two mobile phases eluting at 1.0 mL/min. 3T3-L1 preadipocytes were differentiated into adipocytes for 8 days with or without SBS. After differentiation, accumulated triglyceride contents and leptin production were measured. The correlation coefficients of all constituents in a calibration curve were ≥0.9998 and showed good linearity in the tested concentration range after validation of the method established. The recovery of the four major compounds were 99.46–102.61% with intra- and interday precisions of 0.08–1.01% and 0.15–0.99%, respectively. The four compounds in the lyophilized SBS sample were detected up to 6.46 mg/g. SBS treatment of the differentiated adipocytes significantly inhibited lipid accumulation and leptin production without cytotoxicity. Optimized simultaneous determination of constituents by HPLC–PDA detection will help to improve quality assessment of SBS or related formulas. SBS has an antiadipogenic effect and further investigation to establish the mechanisms of action of its antiadipogenic effect is warranted.

1. Introduction

In general, a traditional herbal medicine prescription is composed of many constituents and at least two herbal medicines. Herbal medicines have been used for treatment and prevention of various diseases for thousands of years (Normile, 2003; Xue and Roy, 2003; Jiang, 2005; Liu et al., 2008). Samryeongbaekchul-san (SBS; Jinryobyakujutsu-san in Japanese and Shenlingbaizhu-san in Chinese) is a well-known traditional herbal medicine prescription, and consists of 12 herbal medicines, Radix Ginseng, Rhizoma Alba Atractylodes, Sclerotium Poria, Rhizoma Dioscoreae, Radix et Rhizoma Glycyrrhizae, Semen Coicis, Semen Nelumbinis, Radix Platycodonis, Semen Dolichoris, Fructus Amomi, Rhizoma Recens Zingiferis, and Fructus Zizyphi in a ratio of 3:3:3:3:3:1.5:1.5:1.5:1.5:1.5:1:1 (Heo, 2007). The main constituents of each raw medicinal herb forming SBS decoction are known as: triterpenoid saponins (e.g. ginsenoside Rb1 and ginsenoside Rg1) from *P. ginseng* (Shan et al., 2014), sesquiterpenoids (e.g. atractylenolide I, II, and III) from *A. japonica* (Tsai et al., 2012), triterpenoids (e.g. pachymic acid and dehydropachymic acid) from *P. cocos* (Li et al., 2004; Hoang et al., 2005), alkaloid (e.g. allantoin) from *D. batatas* (Hwang, 2003), triterpenoids (e.g. glycyrrhizin) and flavonoids (e.g. liquiritin apioside and liquiritin and liquiritigenin) from *G. uralensis* (Zhang and Ye, 2009), phenylpropanoid (e.g. ferulic acid) and flavonoids (e.g. rutin and catechin) from *C. lacryma-jobi* (Wang et al., 2016), flavonoids (e.g. quercetin and kaempferol) from *N. nucifera* (Chen et al., 2012), triterpenoid saponins (e.g. platycodin D) from *P. grandiflorum* (Kim et al., 1990), oleanane-type triterpenes (e.g. chikusetsusaponin IVa and lablabosides A ~ F) from *D. lablab* (Yoshikawa et al., 1998), flavonoid (e.g. quercetin-3-*O*-*β*-$_D$-glucopyranoside) form *A. villosum* (Ying et al., 2014), phenolic compounds (e.g. 6-, 8-, and 10-gingerol) from *Z. officinale* (Zick et al., 2010), and flavonoids (e.g. spinosin and 6‴-feruloylspinosin) from

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Z. jujube (Niu and Zhang, 2011). Recently, the quantitative analysis of the major components in SBS decoction using ultra-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry has been reported (Seo and Shin, 2016). However, this analytical method focuses on the quantitative analysis of the main components in SBS and is a limit to verifying the established method. Biological activities of SBS are reported to have protective effects for irradiated mice (Lee et al., 1999) and a murine model of Alzheimer's disease (Lee, 2009), and an antiosteoporosis effect in an ovariectomized rat model of osteoporosis (Lee, 1998). The effect of SBS on type 2 diabetes and hyperlipemia patients has been reported (Cao et al., 2013). However, research on the antiadipogenic effect of SBS has not yet been reported. Adipogenesis is a cell differentiation process by which preadipocytes become fully differentiated adipocytes. Adipocytes play an important role in lipid metabolism and are closely linked to adipose tissue mass leading to obesity (Otto and Lane, 2005). Because adipogenesis is well characterized by increasing triglyceride deposits in the cytoplasm and secretion of adipokine(s) (Shao et al., 1998; Lefterova and Lazar, 2009; Harris, 2014), targeting these events is considered as a valuable approach to developing new antiobesity agents. In this study, we investigated the inhibitory effect of SBS on adipogenesis *in vitro* and conducted a simultaneous analysis of the main constituents to evaluate the quality of an SBS sample using a high-performance liquid chromatography–photodiode array (HPLC–PDA) detection system.

2. Material and methods

2.1. Plant materials

The 12 raw herbal medicines for the SBS formula shown in Table 1 were purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea) and taxonomically identified by Prof. Young-Bae Seo, College of Oriental Medicine, Daejeon University (Daejeon, Korea) in June 2012. Voucher specimens (2012–KE39–1 through KE39–12) have been deposited at the K-herb Research Center, Korea Institute of Oriental Medicine (KIOM).

2.2. Chemicals and reagents

The reference standards, spinosin (PubChem CID: 155692, purity 98.8%), liquiritigenin (PubChem CID: 114829, purity 99.8%), ginsenoside Rg1 (PubChem CID: 441923, purity 99.3%), and platycodin D2 (PubChem CID: 53317652, purity 98.0%) were purchased from Biopurify Phytochemicals (Chengdu, China). Liquiritin (PubChem CID: 503737, purity 99.6%), ginsenoside Rb1 (PubChem CID: 9898279, purity 98.0%), glycyrrhizin (PubChem CID: 14982, purity 99.0%), and 6-gingerol (PubChem CID: 442793, purity 98.0%) were purchased from Wako Chemicals (Osaka, Japan). Atractylenolide III (PubChem CID: 155948, purity 99.0%), atractylenolide II (PubChem CID: 14448070, purity 99.0%), and atractylenolide I (PubChem CID: 5321018, purity 99.0%) were purchased from KOC Biotech (Daejeon, Korea). Platycodin D (PubChem CID: 162859, purity 98.0%) and allantoin (PubChem CID: 204, purity 98.0%) were purchased from ChemFaces Biochemical (Wuhan, China) and Merck (Darmstadt, Germany). The chemical structures of these reference standard compounds are shown in Fig. 1. The HPLC-grade solvents, methanol, acetonitrile, and water, were purchased from J.T. Baker (Phillipsburg, NJ, USA) and trifluoroacetic acid (TFA, for HPLC, ≥99.0%) was purchased from Merck (Darmstadt, Germany).

2.3. Apparatus and conditions

Simultaneous determination of the main marker compounds in an SBS sample was conducted using a Shimadzu LC-20A series HPLC system (Kyoto, Japan) equipped with a photodiode array (PDA) detector and evaporative light scattering detector (ELSD). The data were measured and processed by Lab Solution software (version 5.53, SP3, Kyoto, Japan). The major constituents were separated on a Phenomenex Gemini C_{18} (250 mm × 4.6 mm; particle size 5 μm, Torrance, CA, USA) and Luna NH_2 column (250 mm × 4.6 mm; particle size 5 μm, Torrance, CA, USA) at a column temperature of 40 °C. The mobile phases consisted of 0.1% (v/v) TFA in distilled water (solvent A) and acetonitrile (solvent B) with gradient elution. The gradient of the two mobile phases was as follows: 10–60% B for 0–30 min, 60–100% B for 30–40 min, 100% B for 40–45 min, and 100–10% B for 45–50 min. The re-equilibration time was 10 min. The mobile phase for the analysis of allantoin consisted of distilled water and acetonitrile, and isocratic elution with 60% acetonitrile for 30 min was used. The analysis was performed at a flow rate of 1.0 mL/min and injection volume of 10 μL.

2.4. Preparations of standard and sample solutions

A standard stock solution of each reference compound was prepared at 1.0 mg/mL using methanol and stored at 4 °C until use. The allantoin stock solution was prepared at the same concentration using water. The SBS decoction was prepared at KIOM. The 12 medicinal herbs as described in Table 1 were mixed and extracted with distilled water at 100 °C for 2 h at 98 kPa pressure using an electric extractor (Cosmos-660; Kyungseo Machine Co., Incheon, Korea). The extracted solution was filtered using a standard sieve (No. 270, 53 μm; Chung Gye Sang Gong Sa, Seoul, Korea) and then the filtered solution was freeze-dried to give a powder

Table 1
Composition of SBS.

Herbal medicine	Scientific name	Family	Origin	Amount (g)
Radix Ginseng	*Panax ginseng* C. A. Meyer	Araliaceae	Yeongju, Korea	11.250
Rhizoma Alba Atrctylodes	*Atractylodes japonica* Koidzumi	Compositae	China	11.250
Sclerotium Poria	*Poria cocos* Wolf	Polyporaceae	Pyeongchang, Korea	11.250
Rhizoma Dioscoreae	*Dioscorea batatas* Decaisne	Dioscoreaceae	Andong, Korea	11.250
Radix et Rhizoma Glycyrrhizae	*Glycyrrhiza uralensis* Fischer	Leguminosae	China	11.250
Semen Coicis	*Coix lacryma-jobi* L. var. *Ma-yuen* Stapf	Gramineae	Muju, Korea	5.625
Semen Nelumbinis	*Nelumbo nucifera* Gaertner	Nymphaeaceae	Vietnam	5.625
Radix Platycodonis	*Platycodon grandiflorum* A. De Candolle	Campanulaceae	Muju, Korea	5.625
Semen Dolichoris	*Dolichos lablab* L.	Leguminosae	China	5.625
Fructus Amomi	*Amomum villosum* Loureiro	Zingiberaceae	Laos	5.625
Rhizoma Recnes Zingiferis	*Zingiber officinale* Roscoe	Zingiberaceae	Ulsan, Korea	3.750
Fructus Zizyphi	*Zizyphus jujube* Miller var. *inermis* Rehder	Rhamnaceae	Yeongcheon, Korea	3.750
Total amount				91.875

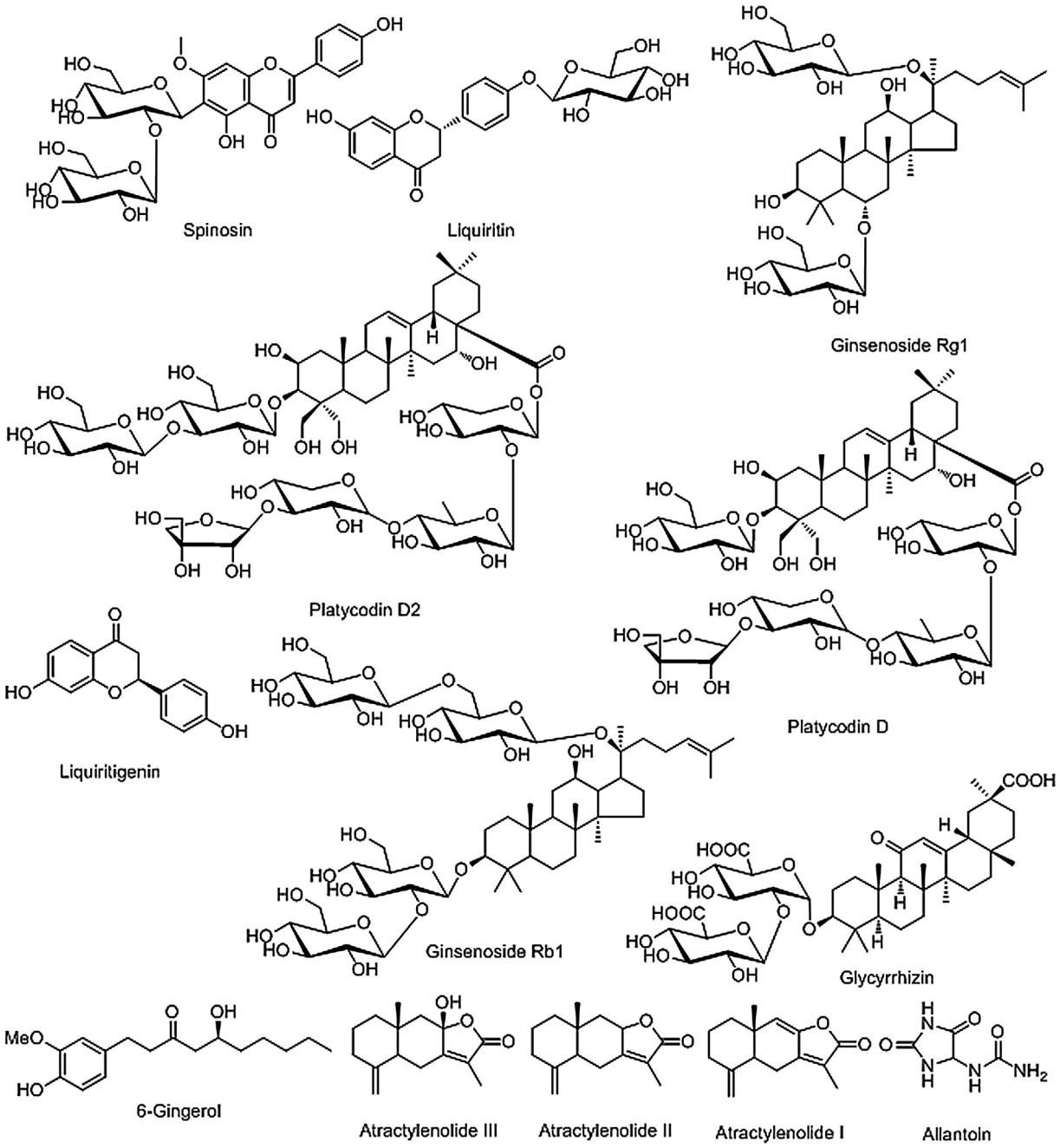

Fig. 1. Chemical structures of the 13 main constituents of SBS.

using a PVT100 freeze dryer (IlShinBioBase, Yangju, Korea). The amount of lyophilized water extract produced was 705.0 g (yield: 14.1%). For quantitative analysis, 200 mg of freeze-dried SBS extract was dissolved in 20 mL of distilled water for 60 min at room temperature using a Branson 8510E-DTH ultrasonicator (Denbury, CT, USA). The extracted solution was filtered through a 0.2 μm membrane (Pall Life Sciences, Ann Arbor, MI, USA) before injection into the HPLC system.

2.5. Method validations

Calibration curves for each reference standard were prepared by plotting peak areas versus the concentration of standard solutions. The linear ranges were 0.78–50.00 μg/mL for spinosin and 6-gingerol, 3.91–250.00 μg/mL for liquiritin and glycyrrhizin, 9.38–300.00 μg/mL for ginsenoside Rb1 and Rg1, 7.81–500.00 μg/mL for platycodin D and D2, 0.39–25.00 μg/mL for liquiritigenin, and 1.56–100.00 μg/mL for atractylenolide I, II, and III. The limits of detection (LOD) and limits of quantification (LOQ) values were calculated as $3.3 \times \sigma/S$ and $10 \times \sigma/S$, respectively (where σ is the standard deviation of the intercept from the regression equation and S is the slope of the calibration curve). Precisions of intra- and interday measurements were confirmed using standard addition method and established by the relative standard deviation (RSD). The repeatability of the optimized analytical method was assessed by RSD values of peak areas and retention times of each analyte after measuring six replicates of the mixed standard solutions. The accuracy test was established by the recovery

experiment and conducted by adding three different concentrations (low, medium, and high) of four reference standards to 200 mg of SBS sample.

2.6. Cell culture and differentiation

The mouse 3T3-L1 preadipocyte cell line was obtained from the American Type Culture Collection (CL-173, ATCC, Rockville, MD). The cells were cultured in DMEM (Gibco BRL, Carlsbad, CA) supplemented with 10% newborn calf serum (Gibco BRL, Carlsbad, CA) at 37 °C. For adipocyte differentiation, the cells were stimulated with 3T3-L1 differentiation medium containing isobutylmethylxanthine, dexamethasone, and insulin (MDI) (Zen-Bio, Research Triangle Park, NC) for 48 h after reaching a confluent state. The medium was switched to DMEM containing 10% FBS and 1 μg/mL insulin after 2 days, and then changed to DMEM containing 10% FBS for an additional 4 days. SBS extract was added to the cell culture during the 8 days of differentiation.

2.7. Cytotoxicity assay

3T3-L1 adipocytes were exposed to various concentrations of SBS extract during 8 days of adipogenesis. CCK-8 solution (Dojindo, Kumamoto, Japan) was added, and the cells were incubated for 4 h. After incubation, the absorbance was read at 450 nm on a microplate reader (Benchmark Plus, Bio-Rad, Hercules, CA).

2.8. Oil Red O staining

The differentiated 3T3-L1 cells were fixed with 10% formalin for 15 min at room temperature and washed with 70% ethanol and PBS. The cells were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO) for 5 min and then washed with PBS. Cell images were collected using an Olympus CKX41 inverted microscope (Olympus, Tokyo, Japan).

2.9. Triglyceride quantification assay

The triglyceride concentration was measured enzymatically using a commercial kit (BioVision, Milpitas, CA). Briefly, the 3T3-L1 adipocytes treated with SBS were homogenized in 5% NP-40 assay buffer, and the sample to solubilize all triglyceride. The samples were heated slowly to solubilize all triglycerides. The sample was mixed with the lipase and triglyceride reaction mixture. After 1 h incubation, the sample absorbance was measured at 570 nm using microplate reader (Benchmark Plus, Bio-Rad. Hercules, CA).

2.10. Leptin immunoassay

Leptin concentration was measured using a mouse leptin immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. In brief, the culture supernatant was collected from differentiated 3T3-L1 adipocytes with or without SBS treatment. Equal amounts of the supernatants (50 μL) and Assay Diluent RD1W (50 μL) were added to a 96-well plate, and the plate was incubated for 2 h at room temperature. The plates were washed five times with 400 μL of wash buffer, 100 μL of mouse leptin conjugate was added to each well, and incubated for 2 h at room temperature. The plates were washed 5 times, 100 μL of substrate solution was added to each well, and incubated for 30 min at room temperature in the dark. Finally, 100 μL of stop solution was added to each well, and the absorbance was measured at 450 nm using microplate reader (Benchmark Plus, Bio-Rad. Hercules, CA).

2.11. Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Group differences were assessed using a one-way ANOVA and Tukey's multiple comparison post hoc test using GraphPad InStat ver. 3.10 (GraphPad Software, San Diego, CA). The differences between the sample and normal control were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Optimization of chromatographic conditions

To separate the 12 main constituents except allantois efficiently, we tested and compared various HPLC conditions including column types (Phenomenex Gemini C_{18}, Waters SunFire C_{18}, and OptimaPak C_{18} column), column temperatures (30, 35, and 40 °C), and mobile phases (acids such as acetic acid, formic acid, and TFA, and an organic solvent with methanol and acetonitrile). In the above conditions, the peak shape, resolution, and baselines of the target compounds were compared. The most suitable conditions for analysis were determined as being a Gemini C_{18} column (250 mm × 4.6 mm, 5 μm), maintained at 40 °C with a mobile phase system of 0.1% (v/v) TFA in distilled water–acetonitrile. The condition for analysis of allantoin was determined as being a Luna NH2 column (250 mm × 4.6 mm, 5 μm) maintained at 40 °C with a mobile phase system of distilled water–acetonitrile. Thirteen constituents were detected at 203 nm for ginsenoside Rb1, ginsenoside Rg1, platycodin D, and platycodin D2, 210 nm for allantoin, 225 nm for atractylenolide II and III, 254 nm for glycyrrhizin, 275 nm for liquiritin, liquiritigenin, and atractylenolide I, 280 nm for 6-gingerol, and 340 nm for spinosin using a PDA detector. All compounds were eluted within 40 min with a resolution ≥ 1.19 (Table 2). Representative HPLC chromatograms of standard compounds and the SBS decoction are shown in Figs. 2 and 3.

3.2. Method validations

The coefficients of determination (r^2) of all constituents were ≥0.9998 and showed good linearity. The LOD and LOQ of all compounds were in the range 0.01–1.61 and 0.03–5.36 μg/mL, respectively. These results showed that the optimized HPLC analytical method was acceptable for the quantitative determination and are summarized in Table 3. The recovery of the four main constituents, liquiritin, liquiritigenin, glycyrrhizin, and allantoin was in the range of 99.46–102.61% and the RSD values did not exceed 1.0% (Table 4). The RSD values of peak responses and retention time for the repeatability test were 0.23–0.73% and 0.02–0.11%, respectively (Table 5), indicating that the established analysis method showed good repeatability. The intra- and interday precision of the four compounds in the SBS samples were evaluated and the RSD values were 0.08–1.01% and 0.15–0.99%, respectively (Table 5). The results demonstrate that the established analytical method using an HPLC–PDA is suitable for quantitative analysis of SBS samples.

3.3. Quantification of SBS decoction

We simultaneously determined the selected 13 main constituents, ginsenoside Rg1 and ginsenoside Rb1 from *P. ginseng*, atractylenolides I, II, and III from *A. japonica*, allantoin from *D. batatas*, liquiritin, liquiritigenin, and glycyrrhizin from *G. uralensis*, platycodin D and platycodin D2 from *P. grandiflorum*, 6-gingerol from *Z. officinale*, and spinosin from *Z. jujube*, in the traditional SBS decoction. Among these constituents, only four compounds,

Table 2
System suitability of the main constituents.

Compound	Capacity factor	Selectivity	Number of theoretical plates	Resolution
Spinosin	3.67	1.07	41142	2.43
Liquiritin	3.91	1.07	32152	2.43
Ginsenoside Rg1	5.11	1.11	133377	8.71
Platycodin D2	5.66	1.01	176365	1.19
Platycodin D	5.74	1.01	132769	1.19
Liquiritigenin	5.99	1.04	79701	2.67
Ginsenoside Rb1	6.76	1.13	215186	9.27
Glycyrrhizin	7.92	1.15	164153	12.82
6-Gingerol	9.14	1.05	156244	4.51
Atractylenolide III	9.64	1.05	126209	4.51
Atractylenolide II	11.25	1.07	245273	8.10
Atractylenolide I	12.07	1.07	261230	8.10
Allantoin	3.67	1.07	41142	2.43

liquiritin, liquiritigenin, glycyrrhizin, and allantoin in the SBS sample were detected and quantified using 210 nm for allantoin, 254 nm for glycyrrhizin, and 275 nm for liquiritin and liquiritigenin with HPLC–PDA detection. The maximum amount of any of the four compounds in the lyophilized SBS sample was 6.46 mg/g, and these results are summarized in Table 6.

3.4. Cytotoxic effect of SBS extract in 3T3-L1 adipocytes

We determined the possible cytotoxicity of the lyophilized SBS sample against 3T3-L1 adipocytes. Preadipose cells were exposed to various concentrations of lyophilized SBS decoction with inducing adipogenesis for 8 days. As shown in Fig. 4A, there was no significant cytotoxic effect up to 1,000 μg/mL treatment of SBS.

3.5. Antiadipogenic effect of SBS extract in 3T3-L1 adipocytes

The effect of lyophilized SBS sample on adipogenesis was evaluated by Oil-Red O staining after differentiating adipocytes by treating then with 400 μg/mL of SBS extract. The lipid droplets detectable with Oil Red O staining were markedly increased in the differentiated adipocytes compared with undifferentiated cells.

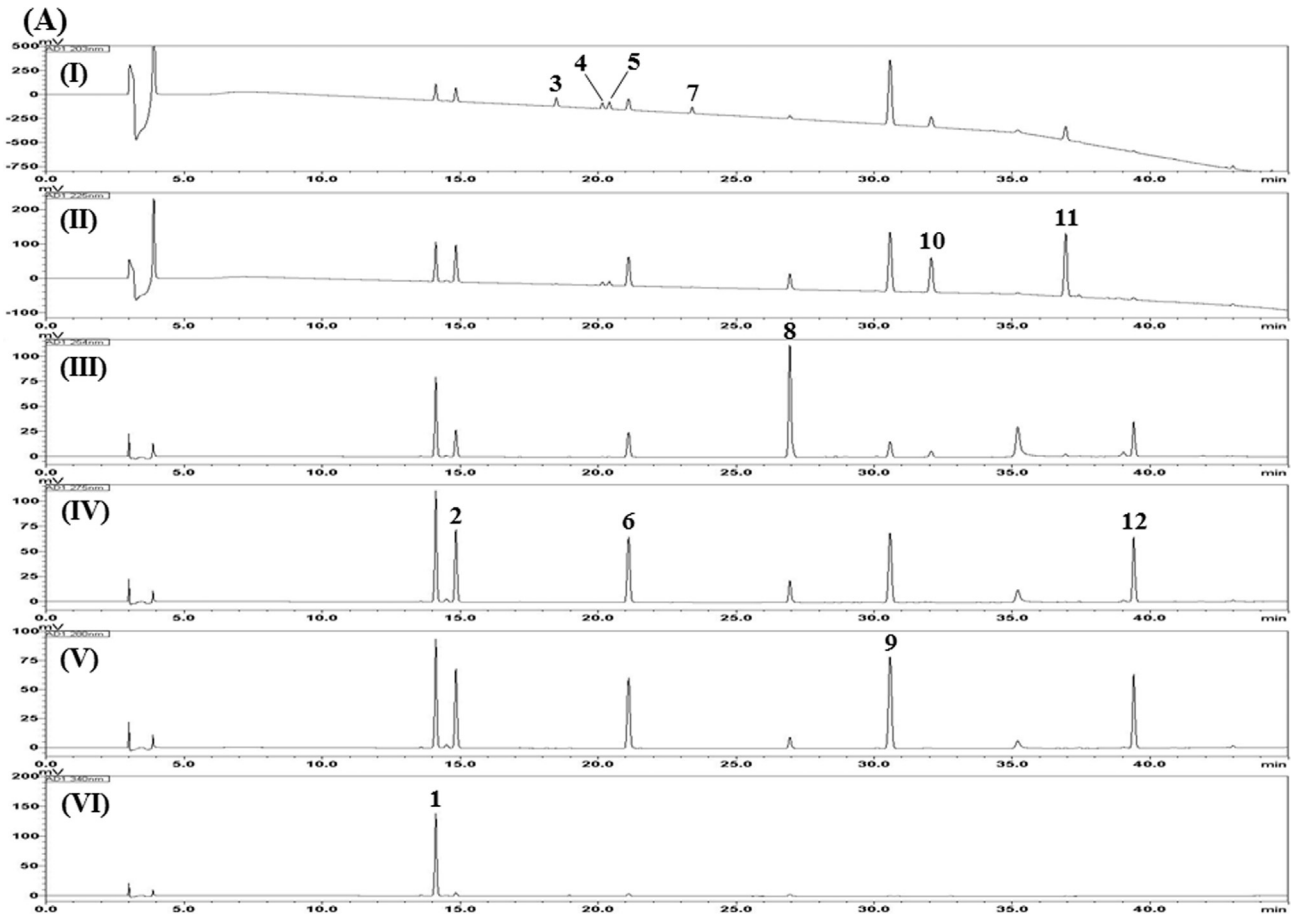

Fig. 2. HPLC chromatograms of standard mixtures (A) and SBS sample (B) at UV wavelength 203 (I), 225 (II), 254 (III), 275 (IV), 280 (V), and 340 (VI) nm. Spinosin (1), liquiritin (2), ginsenoside Rg1 (3), platycodin D2 (4), platycodin D (5), liquiritigenin (6), ginsenoside Rb1 (7), glycyrrhizin (8), 6-gingerol (9), atractylenolide III (10), atractylenolide II (11), and atractylenolide I (12).

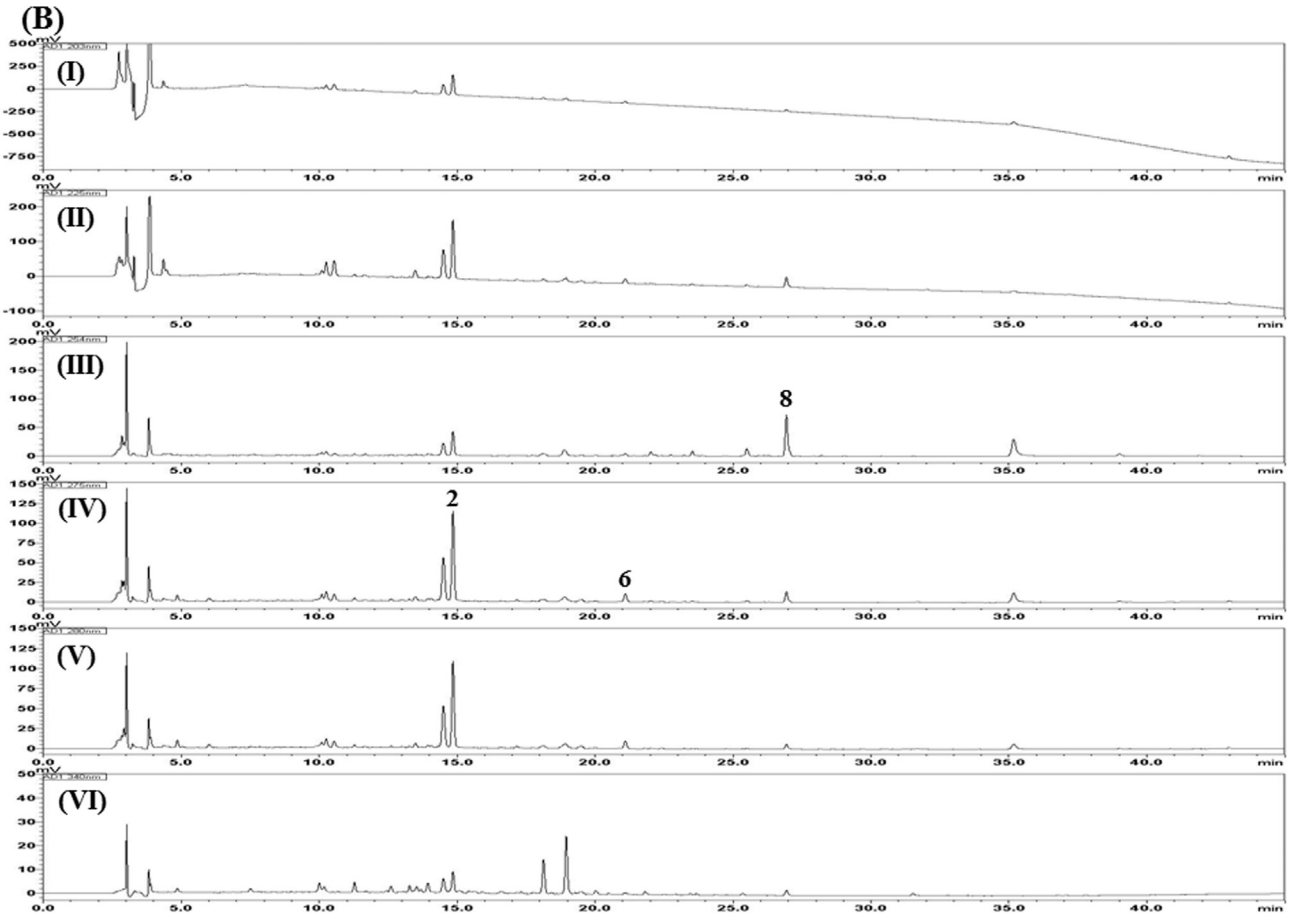

Fig. 2 (*continued*)

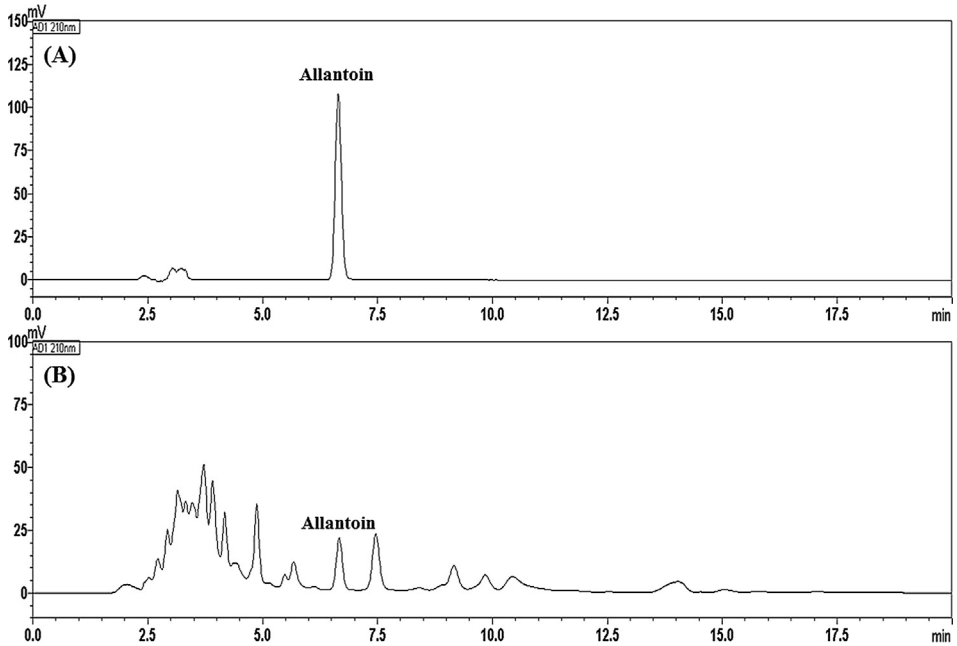

Fig. 3. HPLC chromatograms of allantoin (A) and SBS sample (B) at UV wavelength 210 nm.

Table 3
Linear range, calibration curve, correlation coefficient, LODs, and LOQs for marker compounds ($n = 3$).

Compound	Linear range (μg/mL)	Slope	Intercept	Coefficient of determination (r^2)	LOD[a] (μg/mL)	LOQ[b] (μg/mL)
Spinosin	0.78–50.00	24443.82	−3019.30	0.9998	0.01	0.03
Liquiritin	3.91–250.00	20381.23	−8472.71	0.9999	0.03	0.09
Ginsenoside Rg1	9.38–300.00	2352.24	−1658.61	0.9998	1.56	5.21
Platycodin D2	7.81–500.00	1682.85	−3918.51	0.9999	0.83	2.78
Platycodin D	7.81–500.00	2167.73	−5115.58	0.9999	0.67	2.23
Liquiritigenin	0.39–25.00	38518.74	−1760.91	0.9999	0.04	0.13
Ginsenoside Rb1	9.38–300.00	2234.59	−3960.92	0.9999	1.61	5.36
Glycyrrhizin	3.91–250.00	8998.24	−4246.87	0.9999	0.05	0.17
6-Gingerol	0.78–50.00	6503.80	−786.28	0.9998	0.02	0.06
Atractylenolide III	1.56–100.00	23650.24	−4375.70	0.9998	0.01	0.03
Atractylenolide II	1.56–100.00	38774.68	−12173.95	0.9998	0.01	0.03
Atractylenolide I	1.56–100.00	58782.15	−8114.05	0.9998	0.01	0.03
Allantoin	3.13–100.00	10092.72	4802.62	0.9999	0.03	0.10

[a] $LOD = 3.3 \times \sigma/S$.
[b] $LOQ = 10 \times \sigma/S$.

Table 4
Recoveries for the assay of the four investigated compounds in SBS.

Analytes	Original amount (μg/mL)	Spiked amount (μg/mL)	Detected amount (μg/mL)	Recovery[a] (%)	SD	RSD (%)
Liquiritin	41.25	8.00	49.23	99.69	0.46	0.46
		20.00	61.40	100.13	0.72	0.72
		40.00	81.06	99.52	0.43	0.43
Liquiritigenin	2.15	1.00	3.14	99.46	0.77	0.77
		2.00	4.18	101.61	0.15	0.14
		4.00	6.18	100.96	0.98	0.97
Glycyrrhizin	65.38	12.00	77.37	99.99	0.53	0.53
		30.00	95.63	100.85	0.80	0.79
		60.00	126.61	102.06	0.50	0.49
Allantoin	20.40	4.00	24.41	100.45	0.72	0.71
		10.00	30.42	100.20	0.95	0.95
		20.00	40.92	102.61	0.55	0.53

[a] Recovery (%) = (Original amount − Detected amount)/Spiked amount × 100.

Table 5
Precision of the analytical results (n = 5).

Compound	Spiked Conc. (μg/mL)	Intraday			Interday			Repeatability (n = 6)	
		Detected Conc. (μg/mL)	RSD (%)	Accuracy (%)	Detected Conc. (μg/mL)	RSD (%)	Accuracy (%)	RSD (%) of peak area	RSD (%) of retention time
Liquiritin	8.00	7.96	0.28	99.44	7.99	0.91	99.86	0.73	0.03
	20.00	20.18	0.33	100.89	20.10	0.67	100.49		
	40.00	39.92	0.08	99.80	39.95	0.15	99.88		
Liquiritigenin	1.00	0.99	0.78	83.96	1.00	0.78	84.81	0.35	0.03
	2.00	2.01	0.49	120.75	2.01	0.67	120.59		
	4.00	4.00	0.17	99.92	4.00	0.20	99.92		
Glycyrrhizin	12.00	11.92	0.52	99.31	11.96	0.99	99.64	0.23	0.02
	30.00	29.79	0.55	99.30	29.84	0.73	99.45		
	60.00	60.12	0.12	100.20	60.10	0.16	100.16		
Allantoin	4.00	4.00	1.01	99.90	3.97	0.68	99.23	0.25	0.11
	10.00	9.84	0.55	98.45	9.95	0.77	99.49		
	20.00	20.08	0.10	100.39	20.03	0.18	100.16		

Table 6
Amounts of the four marker compounds in the SBS sample by HPLC ($n = 3$).

Batch No.	Amount (mg/g)											
	Liquiritin			Liquiritigenin			Glycyrrhizin			Allantoin		
	Mean	SD ($\times 10^{-2}$)	RSD (%)	Mean	SD ($\times 10^{-2}$)	RSD (%)	Mean	SD ($\times 10^{-2}$)	RSD (%)	Mean	SD ($\times 10^{-2}$)	RSD (%)
1	3.75	0.49	0.13	0.02	0.06	3.25	6.46	0.34	0.05	1.87	0.52	0.28
2	3.60	1.13	0.31	0.02	0.02	1.23	6.19	2.07	0.33	1.93	5.23	2.71
3	3.72	0.52	0.14	0.02	0.13	7.26	6.44	0.10	0.02	1.89	2.54	1.34
Source[a]	RRG			RRG			RRG			RD		

[a] RRG: Radix et Rhizoma Glycyrrhizae, RD: Rhizoma Dioscoreae.

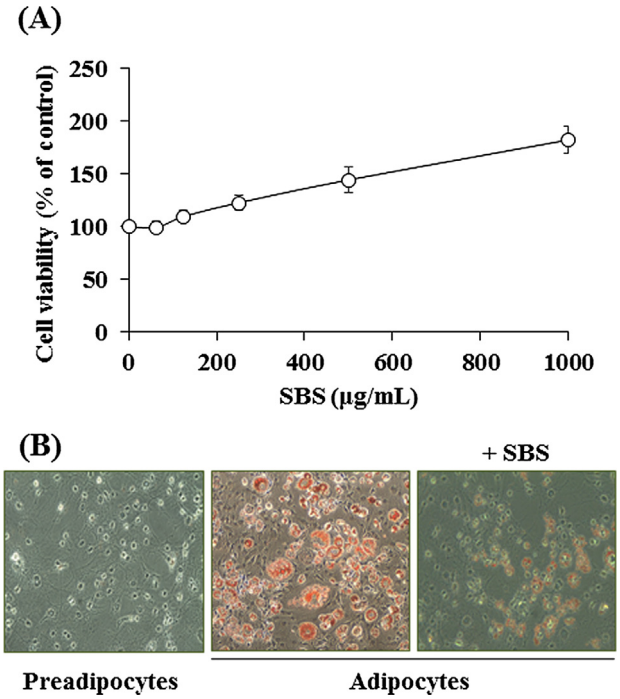

Fig. 4. Cytotoxic and antiadipogenic effects of SBS extract on 3T3-L1 adipocytes. (A) Adipocyte differentiation was induced by adding MDI to 3T3-L1 preadipocytes for 8 days. The cells were exposed to various concentrations of SBS (0, 62.5, 125, 250, 500, or 1000 μg/mL) during the differentiation period. Cell viability was determined using a CCK-8 assay kit by measuring the absorbance at 450 nm. Data are presented as the mean ± SEM. (B) 3T3-L1 adipocytes were exposed to SBS extract (400 μg/mL) for 8 days of adipogenesis. Lipid accumulation in the cells was analyzed by Oil Red O staining. The cells stained with Oil Red O were visualized using an Olympus CKX41 inverted microscope at ×200 magnification. SBS: samryeongbaekchul-san; and MDI: isobutylmethylxanthine, dexamethasone, and insulin.

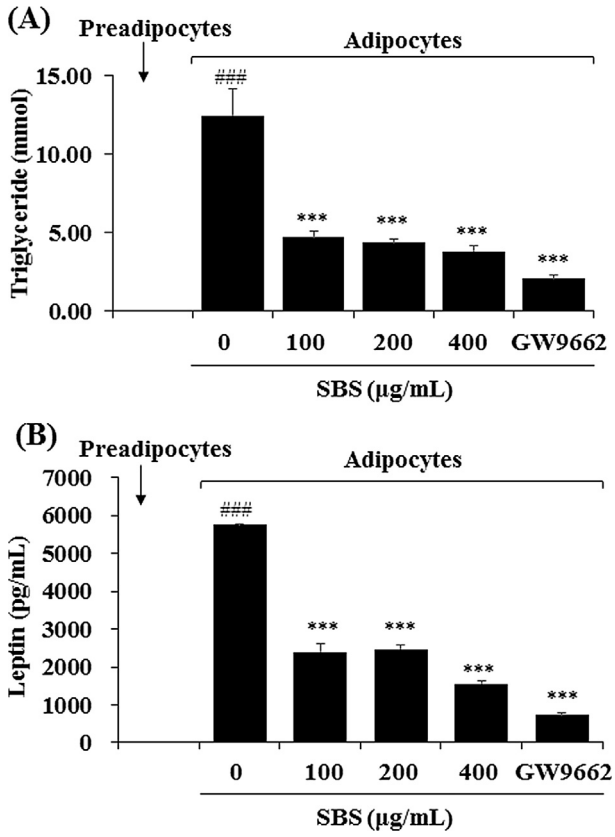

Fig. 5. Inhibitory effects of SBS extract on triglyceride accumulation and leptin production in 3T3-L1 adipocytes. Preadipocytes were differentiated into adipocytes by adding MDI for 8 days. The cells were treated with or without SBS (100, 200, or 400 μg/mL) or GW9662 (20 μM) during the differentiation period. (A) The triglyceride contents were measured enzymatically in cell lysates using a commercial kit (BioVision, Milpitas, CA). (B) The culture supernatant was collected from SBS-treated cells. Leptin production was determined by ELISA using a mouse leptin immunoassay kit (R&D Systems). Data are presented as means ± SEM. $^{\#\#\#}p < 0.001$ vs. undifferentiated adipocytes. $^{***}p < 0.001$ vs. differentiated controls. SBS: samryeongbaekchul-san.

By contrast, SBS treatment of the adipocytes decreased the number of the stained cells compared with untreated adipocytes (Fig. 4B).

3.6. Inhibitory effects of SBS extract on triglyceride production and leptin level in 3T3-L1 adipocytes

To confirm further the antiadipogenic effect of SBS, we measured production of triglyceride and leptin, the key factors in adipogenesis. As shown in Fig. 5A, there was a significant increase in triglyceride content in the differentiated 3T3-L1 cells compared with that in undifferentiated cells. By contrast, SBS extract significantly decreased the amount of triglyceride in 3T3-L1 adipocytes in a dose-dependent manner. Consistently, SBS treatment significantly reduced the adipogenesis-induced leptin production in the adipocytes in a dose-dependent manner (Fig. 5B). GW9662 was used as a positive control (Fig. 5A and B).

4. Conclusion

For the first time to our knowledge, a convenient, accurate, and simple analytical method was established and validated using HPLC–PDA detection for the simultaneous separation and determination of the main constituents in SBS. SBS inhibited lipid accumulation in 3T3-L1 adipocytes by significantly reducing intracellular triglyceride contents without cytotoxicity. Further studies to establish mechanisms of action on the antiadipogenic effect of SBS are warranted.

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