Inhibitory activity of Socheongryong-tang and its constituent components against the production of RANTES, eotaxin, eotaxin-3 and MMP-9 from BEAS-2B cells

JUNG-HOON KIM[1,2*], WOO-YOUNG JEON[1*], MEE-YOUNG LEE[1], CHANG-SEOB SEO[1], HYE-SUN LIM[1] and HYEUN-KYOO SHIN[1]

[1]Herbal Medicine Formulation Research Group, Korea Institute of Oriental Medicine, Daejeon 305-811, Republic of Korea

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Abstract. Socheongryeong-tang (SCRT) is a herbal formula previously used to treat pulmonary diseases primarily caused by the common cold virus, including airway inflammation, asthma and allergy. The aim of the present study was to investigate the inhibitory effect of SCRT water extract and its 13 constituent components on chemokine and enzyme production in the human bronchial epithelium cell line BEAS-2B when induced by tumor necrosis factor-α and interleukin-4. The chemokines examined included regulated on activation of normal T-cell-expressed-and-secreted (RANTES), eotaxin and eotaxin-3. The SCRT water extract demonstrated a dose-dependent inhibition of RANTES, eotaxin, eotaxin-3 and matrix metalloproteinase-9 (MMP-9) in BEAS-2B cells. The 13 constituent compounds of SCRT water extract were quantitatively determined, and it was found that gallic acid, 6-gingerol and methyl eugenol produced the most potent inhibition of RANTES, eotaxin and eotaxin-3 as well as MMP-9 activity regardless of their concentration in SCRT water extract. Principal component analysis and hierarchical clustering analysis revealed that the inhibitory effect of these three compounds contributed to that of SCRT water extract. In conclusion, the results of the present study indicated that the inhibitory effects of SCRT on chemokine and enzyme production in BEAS-2B cells was associated with three of its constituent compounds, gallic acid, 6-gingerol and methyl eugenol. This therefore suggested the potential use of these compounds as anti-inflammatory agents.

Correspondence to: Dr Hyeun-Kyoo Shin, Herbal Medicine Formulation Research Group, Korea Institute of Oriental Medicine, 1672 Yuseongdae-ro, Daejeon 305-811, Republic of Korea
E-mail: hkshin@kiom.re.kr

Present address: [2]Division of Pharmacology, School of Korean Medicine, Pusan National University. Yangsan, Gyeongnam 626-870, Republic of Korea

[*]Contributed equally

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Introduction

Socheongryong-tang (SCRT, Xiaoqinglong-tang in Chinese), is a traditional herbal formula composed of eight herbs: *Ephedrae* herba, *Paeoniae* radix, *Pinelliae* tuber, *Schisandrae* fructus, *Cinnamomi* ramulus, *Asari* radix et rhizoma, *Glycyrrhizae* radix et rhizoma, and *Zingiberis* rhizome. SCRT has been used to treat numerous disorders that can result from improper treatment of the common cold virus, including disturbances to the epigastric region evoked by pathological water stagnation, dry retching, fever and cough with dyspnea (1).

Pharmacological studies of SCRT have demonstrated its protective effects against allergen-sensitized airway inflammation (2,3), histamine signaling in toluene 2,4-diisocyanate-sensitized nasal allergy (4), bleomycin-induced pulmonary fibrosis (5), cockroach allergen-induced asthma (6,7) and elastase-induced chronic obstructive pulmonary disease (8). Numerous studies have reported the efficacy of SCRT water extracts for the treatment of pulmonary diseases; however, its therapeutic mechanism of action remains to be elucidated.

The pharmacological activity of herbal formulas has been associated with the composition of herbal medicines as well as the compounds contained in the herbal formula. Numerous chemical compounds are involved in each herbal medicine; therefore, the therapeutic mechanisms of herbal formulae, complexes of >2 herbal medicines, cannot be elucidated without determining which specific compounds of herbal medicines demonstrate the desired therapeutic effect.

Previous studies have investigated the composition of several herbal medicines in order to elucidate their therapeutic mechanims, including the anticolitis effect of Scutellaria root in Orengedoku-to (9), the activation of peroxisome proliferator-activated receptor alpha in renal cells by *Cinnamomi* cortex in Hachimi-jio-gan (10), the induction of HIV-1 replication by *Ephedrae* herba in Mao-to (11), and the inhibitory effect on murine bone marrow-derived mast cells of *Poria* and *Polyporus* in Saireito and ergosterol, a bioactive compound contained in herbal medicines (12).

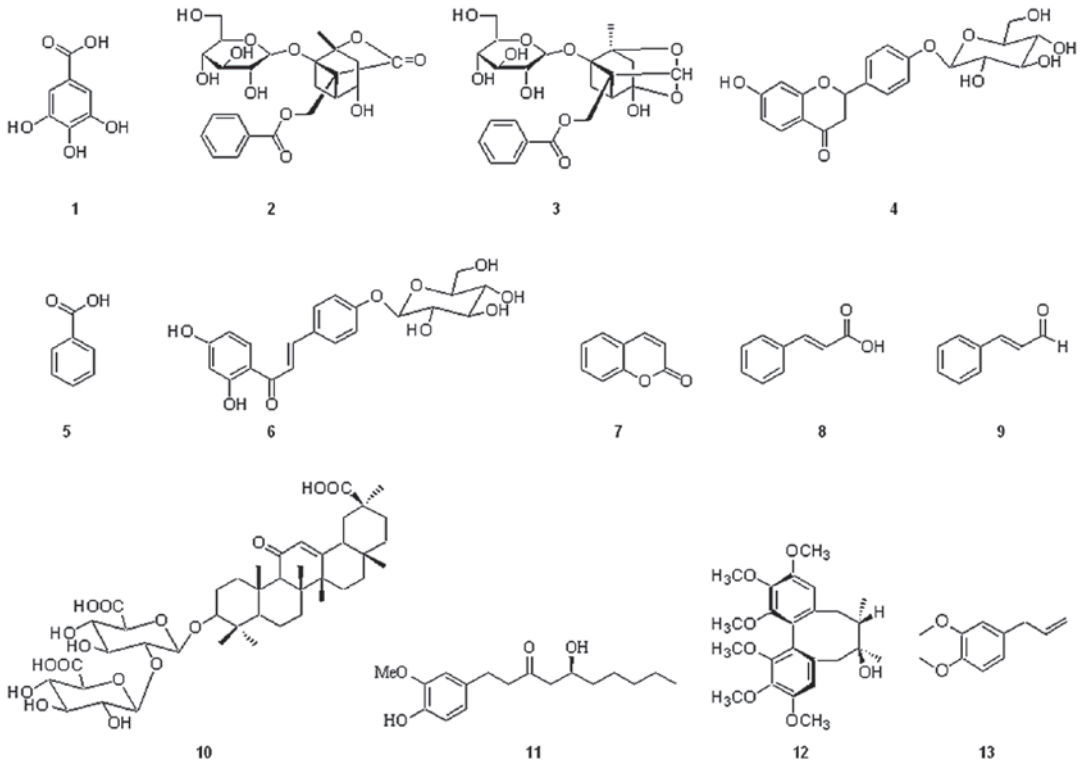

Figure 1. Chemical structures of 13 standard compounds in Socheongryong-tang: 1, gallic acid; 2, albiflorin; 3, paeoniflorin; 4, liquiritin; 5, benzoic acid; 6, isoliquiritin; 7, coumarin; 8, cinnamic acid; 9, cinnamaldehyde; 10, glycyrrhizin; 11, 6-gingerol; 12, schizandrin; and 13, methyl eugenol.

The hypothesis of the present study was that the chemical compounds with the highest quantities in the herbal formulas exert the most predominant therapeutic effect. In the present study, SCRT water extract was prepared and its 13 constituent compounds, gallic acid, albiflorin, paeoniflorin, liquiritin, benzoic acid, isoliquiritin, coumarin, cinnamic acid, cinnamaldehyde, glycyrrhizin, 6-gingerol, schizandrin, and methyl eugenol were detected, as reported in previous literature (13,14), and quantified using high-performance liquid chromatography-diode array detection (HPLC-DAD) and mass spectrometry (MS) methods.

Moreover, the inhibitory effects of SCRT water extract and the 13 compounds were studied *in vitro* using BEAS-2B cells. Assays were used to determine the production of chemokines, including regulated on activation of normal T-cell-expressed-and-secreted (RANTES), eotaxin, and eotaxin-3, which are biomarkers of pulmonary disease, particularly asthma. The possible compounds contributing to the biological effect were confirmed by principal component analysis (PCA) and hierarchical clustering analysis (HCA). These identified compounds were then used to investigate the inhibition of matrix metalloproteinase-9 (MMP-9) in BEAS-2B cells to evaluate the conformational changes caused by asthma and the SCRT.

Materials and methods

Reagents and plant materials. Methanol, acetonitrile and water (HPLC grade) were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Albiflorin, paeoniflorin, cinnamaldehyde, glycyrrhizin, 6-gingerol, and schizandrin were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Gallic acid, benzoic acid, cinnamic acid and methyl eugenol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Liquiritin and isoliquiritin were from NPC BioTech (Geumsan, Korea) and Chengdu Biopurity Phytochemicals Ltd. (Chengdu, China), respectively. All compounds had a purity of >98%. The chemical structures of the standard compounds are shown in Fig. 1. Herbal Medicines were purchased from a Herbal Medicine company, Kwangmyungdang Medicinal Herbs (Ulsan, Korea), and identified by Professor Je-Hyun Lee (Department of Herbology, Dongguk University, Korea). A voucher specimen (2008-KE13-1-8) has been deposited in the Herbal Medicine Formulation Research Group of the Korea Institute of Oriental Medicine.

Preparation of SCRT water extract. A combination of chopped herbal medicines comprising SCRT (total weight, 3.5 kg) (Table I) was extracted with 35 l distilled water at 100˚C for 2 h using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The extracted decoction was filtered using a standard sieve (no. 270; 53 μm; Chunggyesangongsa, Seoul, Korea) and lyophilized to obtain a powder (760.6 g; yield, 21.7%), which was kept at 4˚C until further use for HPLC analysis and *in vitro* assay.

Preparation of standard and SCRT solution. The standard compounds were weighed accurately and dissolved in methanol to make stock solutions at concentrations of 1,000 μg/ml. The stock solution containing a standard compound was diluted to produce working solutions, which were used to construct a calibration curve. Accurately weighed SCRT powder (400 mg) was dissolved in 20 ml distilled water, the solution was then

Table I. Composition of medicinal herbs in Socheongryong-tang.

Herbal medicine	Origin	Amount (g)
Ephedrae herba	China	5.63
Paeoniae radix	Korea	5.63
Pinelliae tuber	China	5.63
Schisandrae fructus	Korea	5.63
Cinnamomi ramulus	Vietnam	3.75
Asari radix et rhizome	China	3.75
Glycyrrhizae radix et rhizome	China	3.75
Zingiberis rhizome	Korea	3.75

filtered through a 0.2-μm syringe filter (SmartPor®; Woongki Science, Seoul, Korea) before it was injected into the HPLC apparatus (Shimadzu LC-20A; Shimadzu, Kyoto, Japan).

Chromatographic conditions. The HPLC system was equipped with a solvent delivery unit (LC-20AT), an autosampler (SIL-20AC), column oven (CTO-20A), degasser (DGU-20A3) and photodiode array detector (SPD-M20A). Separation was performed on a Gemini C18 column (4.6x250 mm, 5 μm particle size of stationary phase; Phenomenex, Torrance, CA, USA) maintained at 40˚C. The mobile phase consisted of water and acetonitrile both containing 1% acetic acid. Gradient elution of the mobile phase was applied: 5-45% at 0-30 min, 45-95% at 30-45 min and held for 5 min. The flow rate was 1.0 ml/min and the injection volume was set to 10 μl. The detection wavelengths were optimized according to the maximum absorption wavelengths of standards (230, 254, 275, 290 and 360 nm).

Method validation. The linear regression and correlation coefficients (r2) of the compounds were calculated on the basis of previously determined calibration curves. The values of limit of detection (LOD) and limit of quantification (LOQ) for each compound were measured using signal concentrations higher than noise concentration at three and ten fold, respectively. The intra-day and inter-day precisions were calculated by analyzing sample extracts spiked with two different concentrations of standard compounds (low and high) and their values were estimated as the relative standard deviation (RSD). Repeatability was evaluated using three replicates of sample solutions that were also represented as the RSD value. To measure the accuracy of the method, a recovery test was performed by adding two different concentration levels of reference compounds (low and high) to the samples, which were then extracted with the above methods. The recovery was calculated as follows: Recovery(%) = ((detected concentration - initial concentration) / spiked concentration) x 100.

Cell culture. A human bronchial epithelial cell line, BEAS-2B, was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL), penicillin (100 U/ml; Gibco-BRL) and streptomycin (100 μg/ml; Gibco-BRL) at 37˚C in an atmosphere of 5% CO_2/95% air under saturating humidity.

Cytotoxicity assay. Cell viability was assessed using the Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. BEAS-2B cells ($6x10^3$ cells/well) were incubated in 96-well plates with various concentrations of SCRT water extract and the 13 constituent compounds for 24 h. CCK-8 reagent was added to each well and incubated for 4 h. The absorbance was measured at 450 nm with a Benchmark Plus microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of cell viability was calculated using the following formula: Cell viability(%)=(mean absorbance in test wells/mean absorbance in vehicle-treated control wells)x100.

Cell stimulation. BEAS-2B cells ($5x10^5$ cells/well) were cultured in six-well plates in medium containing 10% FBS. Once cells had reached confluence, they were washed and incubated with 1 ml serum-free medium containing 10 ng/ml tumor necrosis factor (TNF)-α to produce RANTES for 24 h. Other cells were incubated for 48 h with 50 ng/ml interleukin (IL)-4 + TNF-α (R&D Systems Inc., Minneapolis, MN, USA) to produce eotaxin and eotaxin-3.

Measurement of chemokine production. Culture supernatants were used to measure RANTES, eotaxin and eotaxin-3 using an ELISA (R&D Systems) according to the manufacturer's instructions (Catalog nos. DY278, DY320 and DY346). The absorbance was measured at 450 nm using a Benchmark plus microplate reader (Bio-Rad Laboratories).

Measurement of MMP-9 activity. MMP-9 activity was measured using gelatin zymography. Cell supernatant was mixed with 5X non-reducing sample buffer (Fermentas Inc., Pittsburg, PA, USA) proir to loading onto 10% SDS-PAGE (Bio-Rad Laboratories) containing 1% gelatin as the MMP substrate. The sample was subjected to electrophoresis at 80 volts for 2 h. Following electrophoresis, gels were washed twice in 2.5% Triton X-100 (Sigma-Aldrich) for 1 h to remove SDS and then incubated for 16 h at 37˚C in developing buffer (1 M tris-HCl, pH 7.5; 10 mM $CaCl_2$). Following incubation, gels were stained with Coomassie Brilliant Blue G (Sigma-Aldrich, St. Louis, MO, USA) for 35 min, then de-stained in 25% methanol and 8% acetic acid solution for 20 min and then rinsed twice with de-staining solution to visualize the digested bands in the gelatin matrix. Gelatinase activity was manifested as white bands on a blue background, representing areas of proteolysis of the substrate protein. Images of the gels were captured and the averages of the band intensity were measured using the commercially available ChemiDocTM XRS+ imaging system (Bio-Rad Laboratories).

Statistical analysis. Data are presented as the mean ± standard error of the mean. One-way analysis of variance was used to identify significant differences between the treated and stimulated cells. Dunnett's post-hoc test was used for

Table II. Linear equation, r2, LOD and LOQ for the bioactive compounds in Socheongryeong-tang water extract (n=3).

Compound	Linear equation	r2	Linear range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
Gallic acid	y=32459x-2819.3	0.9999	3.13-50	0.07	0.23
Albiflorin	y=7328x-5019.7	0.9999	3.13-100	0.31	1.04
Paeoniflorin	y=9575.5x+18652	0.9993	14.06-450	0.23	0.76
Liquiritin	y=19804x+25051	0.9994	4.69-150	0.12	0.39
Benzoic acid	y=3219.7x+1404.8	0.9996	3.12-50	0.75	2.49
Isoliquiritin	y=38070x+2477.5	0.9996	0.31-10	0.04	0.12
Coumarin	y=46316x+1860	0.9995	0.31-5	0.05	0.17
Cinnamic acid	y=80734x+9311.1	0.9994	0.47-15	0.03	0.10
Cinnamaldehyde	y=126838x+13957	0.9995	0.49-15.75	0.01	0.03
Glycyrrhizin	y=7478.7x+3063	0.9996	1.88-60	0.19	0.63
6-Gingerol	y=4484.2x+599.19	0.9995	1.88-15	0.51	1.71
Schizandrin	y=21481x+1637.5	0.9996	0.94-30	0.10	0.35
Methyl eugenol	y=9237.6x-296.96	0.9996	1.25-10	0.35	1.16

r2, correlation coefficients; LOD, limit of detection; LOQ, limit of quantification; y, peak area (intensity); x, concentration of compound (μg/ml).

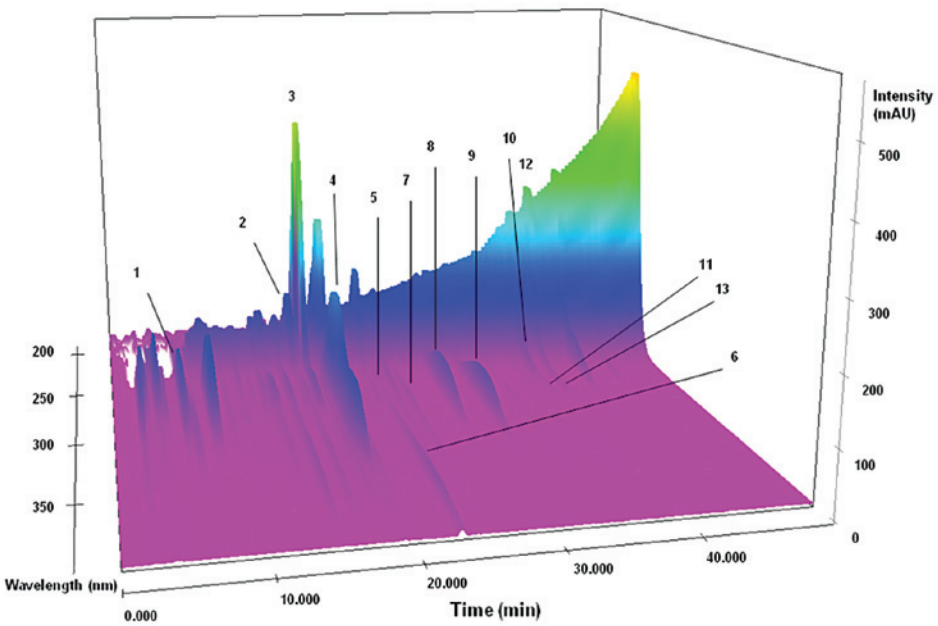

Figure 2. 3D-chromatogram of Socheongryeong-tang water extract at the overall detection wavelength of 13 bioactive compounds: 1, gallic acid; 2, albiflorin; 3, paeoniflorin; 4, liquiritin; 5, benzoic acid, 6, isoliquiritin; 7, coumarin; 8, cinnamic acid; 9, cinnamaldehyde; 10, glycyrrhizin; 11, 6-gingerol; 12, schizandrin; 13, methyl eugenol.

multi-group comparisons. P<0.05 was considered to indicate a statistically significant difference between values. PCA and HCA were performed using a data matrix consisting of rows for the samples (the three concentrations of SCRT water extract and 13 compounds) and columns for the inhibited values of chemokines (RANTES, eotaxin, and eotaxin-3) using open source R software (version 2.15.1; R-project.org).

Results

Method validation. The linear regression and correlation coefficient r2 for each compound ranged from 0.9993 to 0.9999, demonstrating high linearity. The LODs and LOQs were calculated at the concentrations of each compound that produced signal-to-noise ratios of three and ten, respectively; their values were LOD = 0.01-0.75 μg/ml and LOQ = 0.03-2.49 μg/ml (Table II). All compounds were detected in the sample extracts and their chromatograms showed clearly separated maximum absorption wavelengths (Fig. 2). The RSD values for the intraday and interday precision were 0.01-3.21% and 0.20-4.23%, respectively. Repeatability ranged from 0.08-1.99% (Table III). Recovery was used to test the accuracy of the experimental methods. The recovery of each standard compound was 88.30-111.13%, with an RSD of <4.0% (Table IV).

Table III. Intra- and inter-day precision and repeatability of 13 bioactive compounds in the Socheongryeong-tang water extract (n=3).

		Intraday		Interday		
Compound	Spiked concentration (μg/ml)	Detected concentration (μg/ml)	RSD (%)	Detected concentration (μg/ml)	RSD (%)	Repeatability (%)
Gallic acid	9	8.72	1.15	8.74	0.82	0.34
	18	18.14	0.28	18.13	0.20	
Albiflorin	20	20.55	1.33	21.02	2.69	0.66
	40	39.73	0.34	39.49	0.72	
Paeoniflorin	120	126.54	0.77	126.64	1.28	0.45
	240	236.73	0.21	236.54	0.42	
Liquiritin	30	28.28	0.97	28.25	0.82	0.08
	60	60.86	0.23	61.20	1.13	
Benzoic acid	10	9.49	2.32	9.54	1.24	1.26
	20	20.26	0.54	20.43	1.88	
Isoliquiritin	2	2.02	1.74	2.03	1.91	0.24
	4	3.99	0.44	3.98	0.49	
Coumarin	1	1.02	1.13	1.03	1.32	0.35
	2	1.99	0.29	1.99	0.34	
Cinnamic acid	3	2.95	0.05	2.96	1.10	0.14
	6	6.02	0.01	6.05	0.98	
Cinnamaldehyde	2.1	2.00	0.49	1.98	0.40	0.65
	4.2	4.25	0.12	4.25	0.43	
Glycyrrhizin	12	11.77	0.13	11.94	1.73	0.25
	24	24.11	0.03	24.05	0.51	
6-Gingerol	2	2.09	3.21	2.09	3.53	1.45
	4	3.95	0.85	3.94	1.06	
Schizandrin	5	5.04	1.13	5.01	0.99	1.99
	10	9.98	0.28	10.04	4.23	
Methyl eugenol	1	1.06	2.59	1.08	2.63	1.22
	2	1.97	0.70	1.96	0.73	

RSD, relative standard deviation; (%) = (standard deviation/mean) x100.

Abundance of the constituent compounds in the SCRT water extract. The 13 constituent compounds were quantified in the SCRT water extract. Paeoniflorin was the most abundant compound in the SCRT water extract, followed by liquiritin, albiflorin and glycyrrhizin, while methyl eugenol was observed at the lowest quantity of ~100 times lower than that of paeoniflorin (Table V).

SCRT water extract inhibits chemokine production. Chemokine levels, including RANTES, eotaxin and eotaxin-3 in BEAS-2B cells were determined using ELISA following treatment with SCRT water extract. Nontoxic concentrations of samples were used for the subsequent experiments by the determination of the cytotoxicity on BEAS-2B cells (data not shown). The production of RANTES was significantly increased following treatment with TNF-α; however, following treatment with SCRT water extract, production was reduced in a dose-dependent manner (Fig. 3A). Cells treated with IL-4 + TNF-α exhibited significantly increased secretion of eotaxin and eotaxin-3. However, SCRT water extract markedly decreased the production of eotaxin and significantly attenuated the production of eotaxin-3 in a dose-dependent manner (Fig. 3B and C).

The 13 constituent compounds of SCRT inhibit chemokine production. The production of chemokines was measured in BEAS-2B cells treated with the constituent compounds of SCRT in order to identify the specific compounds which may contribute to its inhibitory effect. With the exception of isoliquiritin, all compounds were found to significantly suppress the increased productions of RANTES in TNF-α-treated BEAS-2B cells in a dose-dependent manner (Fig. 4A). In addition, eotaxin-3 production was significantly and dose-dependently inhibited by the treatment with eight of the compounds, including gallic

Table IV. Recovery of 13 bioactive compounds in the Socheongryeong-tang water extract (n=3).

Compound	Initial concentration (μg/ml)	Spiked concentration (μg/ml)	Detected concentration (μg/ml)	Recovery (%)	RSD (%)
Gallic acid	19.85	9	28.85	106.00	0.42
		18	37.85	113.13	0.74
Albiflorin	42.45	20	62.45	104.45	1.16
		40	82.45	96.53	0.38
Paeoniflorin	244.47	120	364.47	99.01	0.36
		240	484.47	93.86	1.87
Liquiritin	62.62	30	92.62	93.16	2.77
		60	122.62	99.18	1.69
Benzoic acid	19.49	10	29.49	102.41	0.92
		20	39.49	109.33	1.76
Isoliquiritin	4.81	2	6.81	102.81	0.35
		4	8.81	98.85	0.32
Coumarin	4.31	1	5.31	101.28	2.39
		2	6.31	98.22	0.81
Cinnamic acid	6.76	3	9.76	99.41	0.40
		6	12.76	101.98	0.28
Cinnamaldehyde	4.72	2.1	6.82	88.83	0.57
		4.2	8.92	96.01	0.50
Glycyrrhizin	40.50	12	52.5	105.99	0.17
		24	64.5	109.09	0.27
6-Gingerol	9.12	2	11.12	96.98	3.89
		4	13.12	91.20	1.71
Schizandrin	11.91	5	16.91	110.52	1.24
		10	21.91	108.62	1.99
Methyl eugenol	2.47	1	3.47	104.19	3.01
		2	4.47	93.25	2.85

RSD, relative standard deviation; (%) = (standard deviation/mean) x100.

acid, paeoniflorin, liquiritin, benzoic acid, couomarin, cinnamic acid, 6-gingerol and methyl eugenol, whereas only three compounds, gallic acid, 6-gingerol and methyl eugenol, reduced the release of eotaxin in BEAS-2B cells pretreated with IL4 and TNF-α (Fig. 4B and C).

The influence of constituent compounds on the inhibition of chemokine production. To evaluate the influence of constituent compounds on the inhibition of chemokines, multivariate statistical analysis, including PCA and HCA, were performed.

PCA was represented in a plot consisting of two principal component (PC) scores. PC1 contributed 71% of the total variance, and therefore is a highly significant variable for the distribution of samples. Three concentrations (low, medium and high) of SCRT water extract, 6-gingerol and methyl eugenol, two levels (medium and high) of gallic acid, and high levels of albiflorin, liquiritin, isoliquiritin, benzoic acid, cannamic acid, cinnamaldehyde and schizandrin, were distributed at the negative PC1 score. Samples were clearly classified by PC1 score. PC2, the next most significant variable of distribution of samples orthogonal to PC1, contributed 18% of the total variance. The samples with negative PC1 scores consisted of three concentrations of SCRT water extract, two concentrations of 6-gingerol (medium and high) and methyl eugenol (low and medium), and high concentrations of coumain were assigned positive PC2 score. All arrows of chemokines (RANTES, eotaxin and eotaxin-3) were directed at negative PC1 score, with RANTES and eotaxin assigned a negative PC2 score, whereas eotaxin-3 was assigned a positive PC2 score (Fig. 5).

HCA represented the distribution of the concentrations of SCRT water extract and its 13 constituent compounds as a dendrogram. The results exhibited a similar pattern of classification to that of the PCA. Two main groups, A and B, were classified at a height of ~400. Three concentrations of SCRT water extract, two concentrations (medium and high) of gallic acid, 6-gingerol, and methyl eugenol, and a high concentration of isoliquiritin were involved in group A, which was clearly distinct from group B (Fig. 6).

Table V. Average content of 13 compounds in the Socheongryeong-tang water extract (n=3).

Compound	Average content (mg/g)
Gallic acid	0.993±0.004
Albiflorin	2.123±0.010
Paeoniflorin	12.224±0.016
Liquiritin	3.131±0.034
Benzoic acid	0.974±0.013
Isoliquiritin	0.240±0.000
Coumarin	0.215±0.000
Cinnamic acid	0.338±0.000
Cinnamaldehyde	0.236±0.002
Glycyrrhizin	2.025±0.006
6-Gingerol	0.456±0.001
Schizandrin	0.596±0.003
Methyl eugenol	0.123±0.000

Average content represented as the mean ± standard deviation.

SCRT water extract and its active components inhibit the activities of MMP-9. Gelatin zymography was used to investigate the effect of SCRT water extract, gallic acid, 6-gingerol and methyl eugenol on MMP-9 activity in BEAS-2B cells induced with IL4 and TNF-α. The purpose of this assay was to elucidate the inhibitory mechanism of SCRT against airway-associated inflammation. As shown in Fig. 7, MMP-9 activity was markedly increased following treatment of BEAS-2A cells with IL4 and TNF-α; by contrast, MMP-9 activity was significantly reduced following treatment with SCRT water extract in a dose-dependent manner. It was also observed that gallic acid, one of the 3 contributing compounds, dose-dependently inhibited the increased MMP-9 activity (Fig. 7A and C). In addition, high levels of 6-gingerol significantly inhibited MMP-9 activity and methyl eugenol demonstrated a non-significant reduction of increased MMP-9 activity (Fig. 7B and D).

Discussion

The BEAS-2B cell line derived from human bronchial epithelial normal cells has been used to examine endotoxin- or cytokine-associated airway inflammation that may consequentially induce allergic asthma (15,16). Following stimulation with lipopolysaccharide, TNF-α, IL-4 or IL-31, BEAS-2B cells are reported to secrete pathologically elevated levels of chemokines and cytokines, including RANTES, IL-8, eotaxin, epidermal growth factor, vascular endothelial growth factor and monocyte chemoattractant protein-1 (17-20).

RANTES, a member of a large supergene family of proinflammatory cytokines, is secreted by macrophages, platelets, fibroblasts, vascular endothelial cells, lymphocytes and epithelial cell lines; RANTES production is enhanced by pretreatment with cytokines including IL-5, TNF-α and interferon-γ (IFN-γ), and it functions as a strong inducer of eosinophil transendothelial migration (21,22). Eotaxin is an eosinophil-specific cystein-cystein

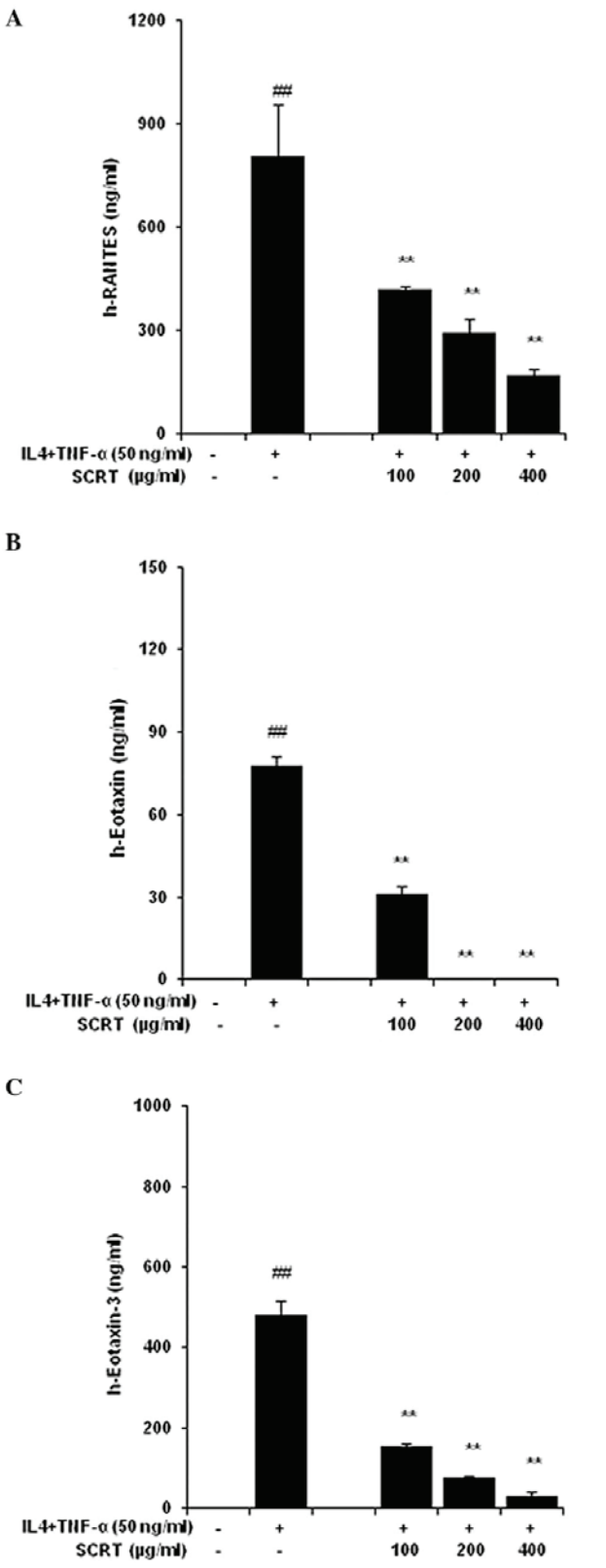

Figure 3. Effects of SCRT water extract on the production of RANTES, eotaxin and eotaxin-3 in BEAS-2B cells. Cells were treated with SCRT water extract and then co-stimulated with 10 ng/ml TNF-α (RANTES) or 50 ng/ml each of TNF-α + IL-4 (eotaxin and eotaxin-3) for 24 or 48 h, respectively. The levels of (A) RANTES, (B) eotaxin and (C) eotaxin-3 released into the culture medium were assessed using commercially available ELISA kits. $^{\#\#}$P<0.01 versus vehicle control group; *P<0.05 and **P<0.01 versus TNF-α or TNF-α + IL-4-treated cells. SCRT, Socheongryeong-tang; RANTES, regulated on activation of normal T-cell-expressed-and-secreted; TNF-α, tumor necrosis factor α; IL-4, interleukin 4; h, human-oriented.

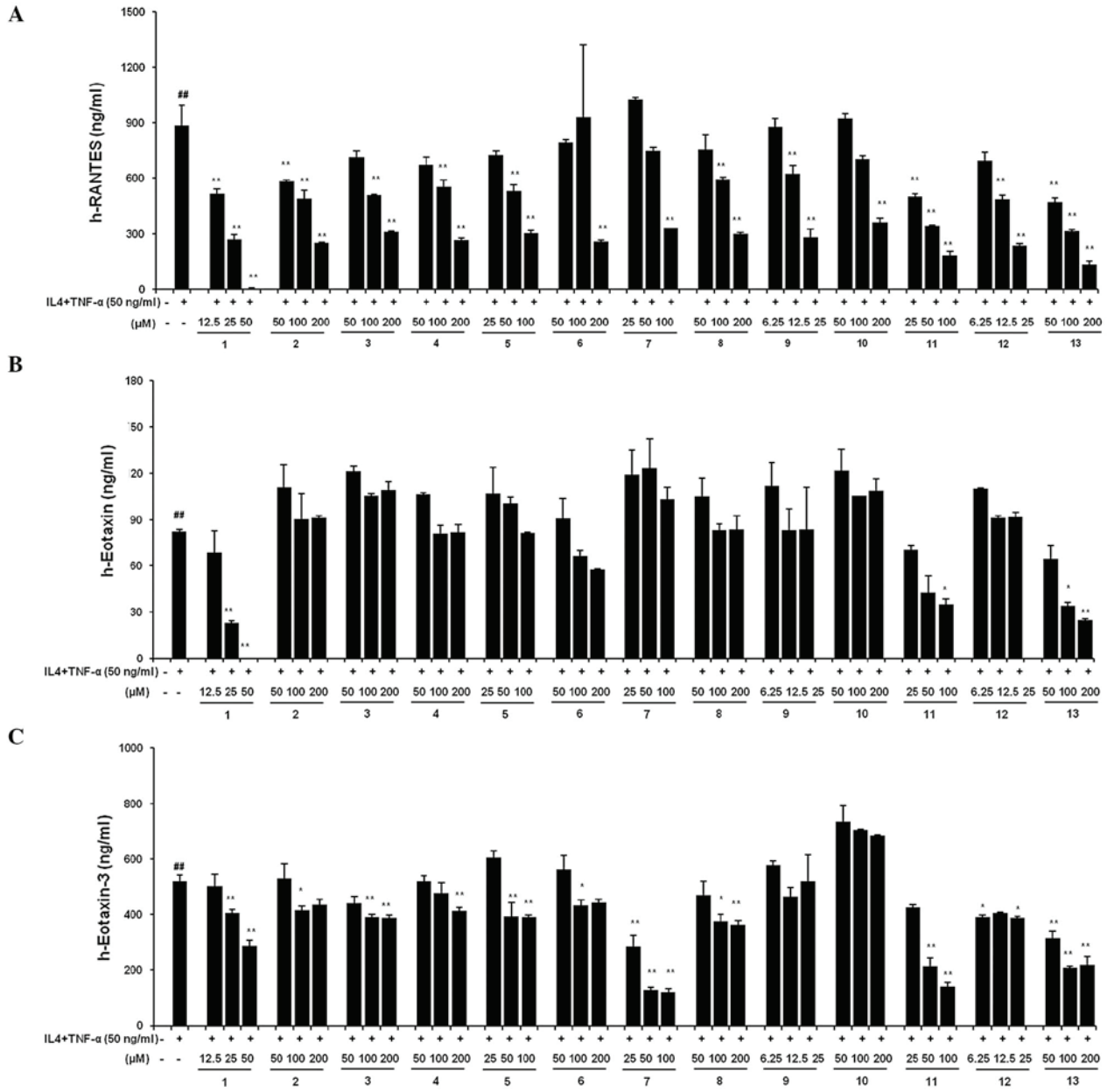

Figure 4. Effects of the 13 constituent compounds of Socheongryeong-tang water extract on the production of RANTES, eotaxin, and eotaxin-3 in BEAS-2B cells. Cells were treated with the 13 constituent compounds: 1, gallic acid; 2, albiflorin; 3, paeoniflorin; 4, liquiritin; 5, benzoic acid; 6, isoliquiritin; 7, coumarin; 8, cinnamic acid; 9, cinnamaldehyde; 10, glycyrrhizin; 11, 6-gingerol; 12, schizandrin; and 13, methyl eugenol and then co-stimulated with 10 ng/ml TNF-α (RANTES) or 50 ng/ml each of TNF-α + IL-4 (eotaxin and eotaxin-3) for 24 or 48 h, respectively. The levels of (A) RANTES, (B) eotaxin and (C) eotaxin-3 released into the culture medium were assessed using commercially available ELISA kits. $^{\#\#}$P<0.01 versus vehicle control group; *P<0.05 and **P<0.01 versus TNF-α or TNF-α + IL-4-treated cells. RANTES, regulated on activation of normal T-cell-expressed-and-secreted; TNF-α, tumor necrosis factor α; IL-4, interleukin 4.

(CC) chemokine produced by airway epithelial cells, smooth muscle cells and fibroblasts; it is also a potent chemoattractant that mobilizes eosinophils into the airway following allergic stimulation. Production of eotaxin is known to be enhanced by T-helper cell 2 (Th2) cytokines such as IL-4 and IL-13 (17,23). Eotaxins represent a group of chemokines consisting of three sets of subtypes: Eotaxin-1 (CC ligand 11; CCL11) is secreted by eosinophils, macrophages, lymphocytes, fibroblasts, smooth muscle and endothelial cells, while eotaxin-2 (CCL24) and eotaxin-3 (CCL26) are mainly released by epithelial and endothelial cells in greater amounts than eotaxin-1 (24). Eotaxin-3 binds to CC receptor 3, a G protein-coupled receptor that is highly expressed in eosinophils, acts as a potent eosinophil chemoattractant and is upregulated in human bronchial epithelial cells when stimulated by Th2 cytokines, including IL-4 and IL-13 (25,26). In asthma, eosinophils are recruited to an inflammatory site, increasing airway inflammation and immune-regulation, including the release of cytotoxic granules, lipid mediators, as well as the production of cytokines and chemokines, which consequently cause bronchoconstriction, mucus secretion and lung remodeling (27). Therefore, the inhibition of RANTES, eotaxin and eotaxin-3 secretions from BEAS-2B cells may help to relieve airway inflammation due to overproliferation of eosinophils as they are pivotal for eosinophil chemotaxis and are

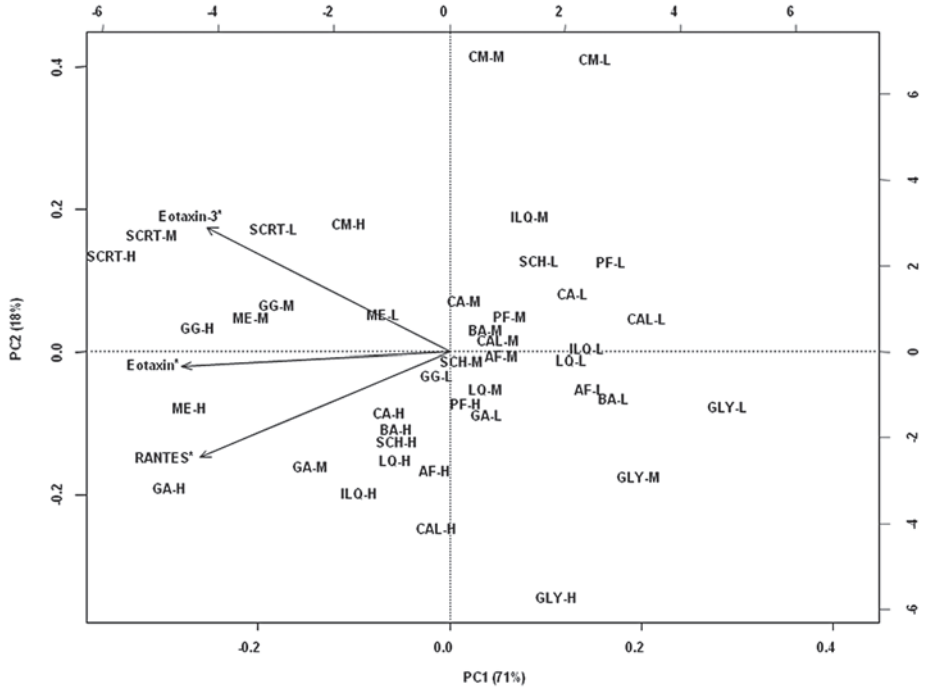

Figure 5. Biplot of principal components (PC1 vs. PC2) on the variables (percentage inhibition of chemokine production) with the objectives (three concentration levels of SCRT water extract and its 13 constituent compounds). The effects of chemokines on constituent compounds are represented by arrows. PC1 and PC2 contributed to 71% and 18% of total variance, respectively. SCRT, Socheongryeong-tang water extract; GA, gallic acid; AF, albiflorin; PF, paeoniflorin; LQ; liquiritin; BA, benzoic acid; ILQ, isoliquiritin; CM, coumarin; CA, cinnamic acid; CAL, cinnamaldehyde; GLY, glycyrrhizin; GG, 6-gingerol; SCH, schizandrin; ME, methyl eugenol; L, low concentration; M, medium concentration; H, high concentration; PC, principal component.

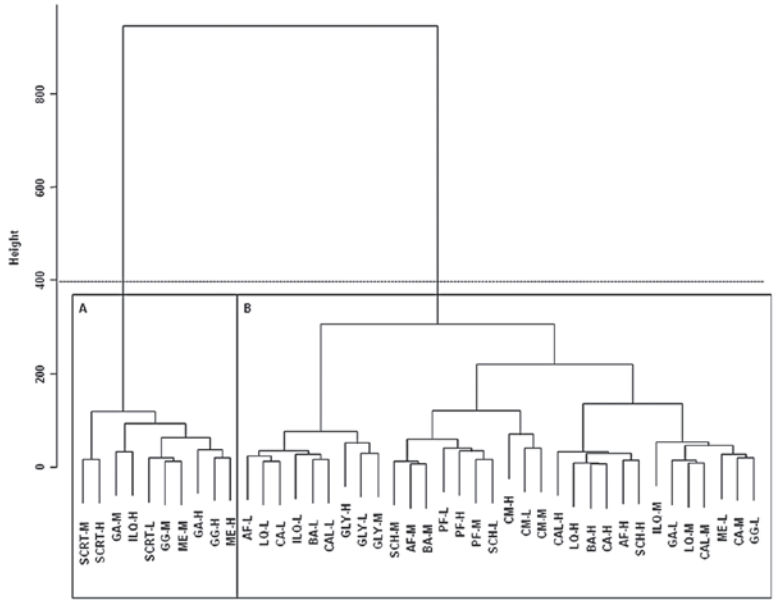

Figure 6. Dendrogram of the classification of SCRT water extract and 13 constituent compounds with the three levels of concentrations (low, medium and high) administered to BEAS-2B cells. Two groups, A and B, were separated at the height of 400. Ward's method was applied using Euclidean distance for the classification. SCRT, Socheongryeong-tang water extract; GA, gallic acid; AF, albiflorin; PF, paeoniflorin; LQ; liquiritin; BA, benzoic acid; ILQ, isoliquiritin; CM, coumarin; CA, cinnamic acid; CAL, cinnamaldehyde; GLY, glycyrrhizin; GG, 6-gingerol; SCH, schizandrin; ME, methyl eugenol; L, low concentration; M, medium concentration; H, high concentration.

significantly correlated with eosinophil recruitment and activation (22).

The therapeutic effect of SCRT in treating pulmonary diseases is considered beneficial in Traditional Medicine as well as modern pharmacology. The BEAS-2B cell line was used to investigate the inhibitory effect of SCRT on airway inflammation. It was hypothesized that the chemical compounds with the highest quantities in the herbal formulas would exert the most predominant inhibitory effect on inflammatory mediators; however, herbal medicines consist

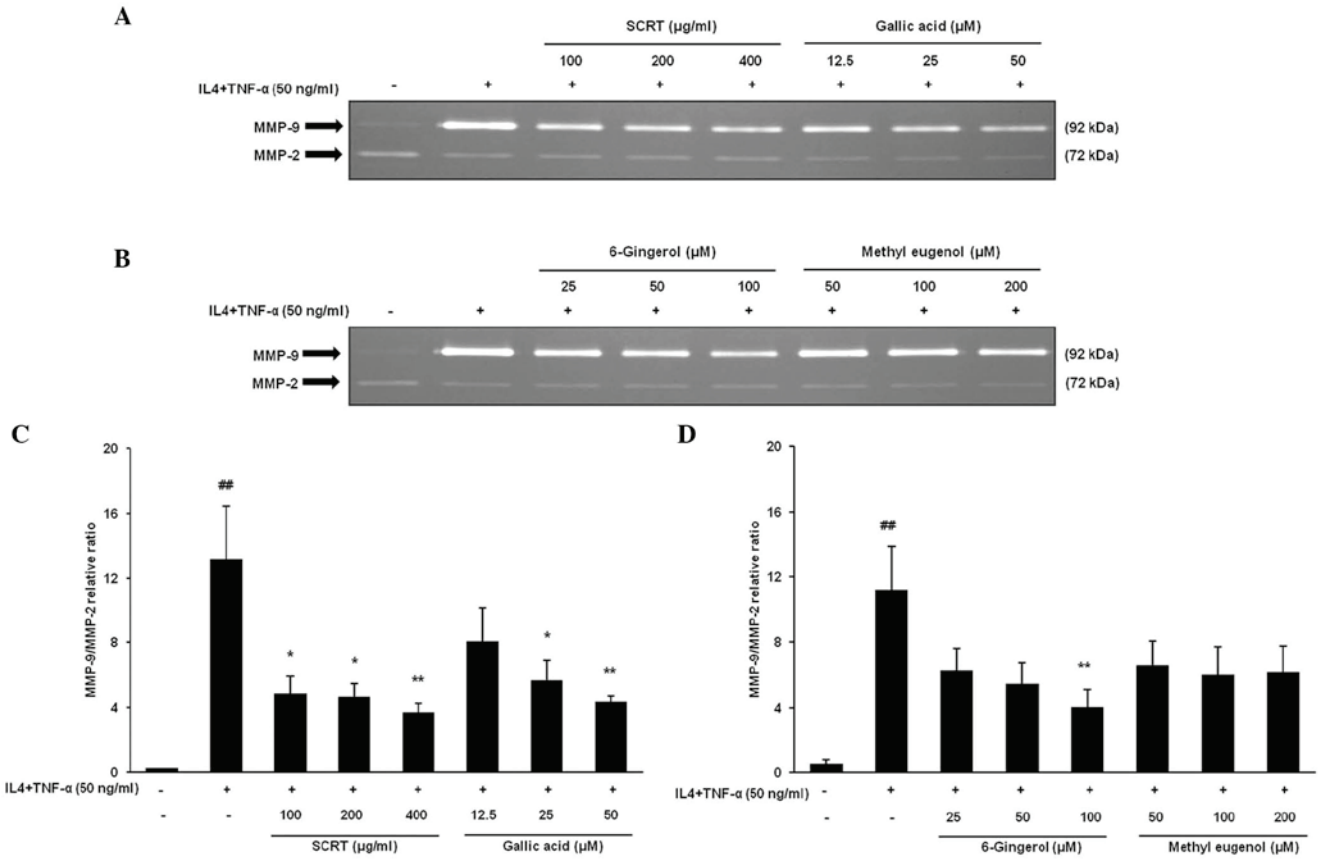

Figure 7. Effects of the SCRT water extract and 3 constituent compounds, gallic acid, 6-gingerol and methyl eugenol on MMP-9 activity in BEAS-2B cells. Cell supernatants were loaded for gelatin zymography. (A and B) Representative photographs of MMP-9 activities and band intensity of MMP-9. (C and D) Relative ratio of MMP-9 activities normalized to MMP-2 were quantitated. $^{\#\#}$P<0.01 versus vehicle control group; *P<0.05 and **P<0.01 versus TNF-α or TNF-α + IL-4 treated cells. SCRT, Socheongryeong-tang; TNF-α, tumor necrosis factor α; IL-4, interleukin 4; MMP, matrix metalloproteinase.

of numerous compounds and their biological effect may be attributed to synergic actions of the constituent compounds. To find the most potent compounds for the inhibition of chemokine production, the 13 compounds were quantitatively analyzed in order to determine their amounts in SCRT water extract. The suppressive capacities of these 13 compounds on the secretion of RANTES, eotaxin and eotaxin-3 were evaluated in BEAS-2B cells. The BEAS-2B cell line was induced using TNF-α or TNF-α in combination with IL-4, as it was reported that their combination may enhance the production of eotaxin in human lung fibroblasts, airway epithelial cells, smooth muscle cells and dermal fibroblasts (28).

Dose-dependent inhibition of secretions of RANTES, eotaxin and eotaxin-3 by SCRT water extract was observed in BEAS-2B cells. However, in contrast to the hypothesis of the present study, the more abundant compounds, including albiflorin, paeoniflorin, liquiritin and glycyrrhizin, did not potently inhibit the production of RANTES, eotaxin and eotaxin-3 in BEAS-2B cells. Conversely, gallic acid, 6-gingerol and methyl eugenol demonstrated the most prominent inhibition of chemokine production, although these compounds, particularly methyl eugenol, were not found in markedly higher quantities than others in the SCRT water extract. These results were supported by literature which reported the decrease of eotaxin following treatment of 6-gingerol in inflammation (29). The results of the present study implied that the inhibitory effect on chemokine production from BEAS-2B cells may not be ensured by the constituent compounds with higher contents but the compounds that were more specific to cell lines or chemokines, regardless of their quantity.

Furthermore, PCA and HCA were used to investigate the association between objectives (SCRT water extract and constituent compounds) and variables (percentage inhibition of each chemokine secretion). PCA was represented by PC scores that contributed highly to the differentiation of samples; the closer the PC scores, the closer the association (30). The combination of the two PCs enabled the samples to be more differentiated; therefore, the two PCs described 89% of the total variance, which may explain almost the entire correlation between samples. Three concentrations of SCRT water extract, 6-gingerol and methyl eugenol, two concentrations of gallic acid, and high concentrations of albiflorin, liquiritin, isoliquiritin, benzoic acid, cannamic acid, cinnamaldehyde and schizandrin were all located at the negative PC1 score and therefore were considered to have a closer association than the samples with a positive PC1 score. Of the variables with negative PC1 score, SCRT (low, medium and high concentrations), 6-gingerol (medium and high concentrations), methyl eugenol (low and medium concentrations) and coumarin (high concentration) were positioned at the positive PC2 score demonstrating a closer association with the variables of the negative PC2 score. HCA, a multivariate analysis method used to classify samples into groups providing

a visual representation of complex data (31), created two distinguishable groups. The variables, including SCRT water extract, gallic acid, 6-gingerol, methyl eugenol and isoliquiritin were contained in group A, indicating a strong association with each other. The results of the PCA and HCA indicated that the inhibitory effect of three compounds, including gallic acid, 6-gingerol and methyl eugenol, were closely associated with that of SCRT water extract and may therefore be contributing factors in the suppression of chemokine production in BEAS-2B cells.

The suppressive effect of SCRT water extract and three active components (gallic acid, 6-gingerol, and methyl eugenol) was further investigated to examine the inhibition of MMP-9 in BEAS-2B cells. MMPs are an enzyme family which degrade the extracellular matrix and basement membrane, infiltrate inflammatory cells and consequently participate in tissue remodeling (32). MMP-9 decomposes type IV and V collagen as well as elastin, and therefore has an important role in airway inflammation (33,34). MMP-9 levels were reported to be elevated in pulmonary diseases such as chronic obstructive pulmonary disease (35). SCRT water extract and the three contributing compounds suppressed the increase of MMP-9 activity induced by IL-4 and TNF-α; however, 6-gingerol and methyl eugenol did not demonstrate a statistically significant inhibition. This result indicated that SCRT and the 3 compounds attenuated the inflammatory response of BEAS-2B cells through inhibition of MMP-9 activity.

In conclusion, the present study showed that SCRT had an inhibitory effect on the production of chemokines, including RANTES, eotaxin and eotaxin-3, as well as MMP-9 in BEAS-2B cells and this inhibitory effect of SCRT could not be associated to the concentration of the constituent compounds but to the specificity of a compound to affect chemokines and enzymes. The production of RANTES, eotaxin and eotaxin-3 as well as MMP-9 from BEAS-2B cells was significantly suppressed by the compounds gallic acid, 6-gingerol and methyl eugenol, even when present at low or undetectable levels in the SCRT water extract, which may contribute to the anti-inflammatory activity of SCRT.

Acknowledgements

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