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삼출건비탕의 HPLC-PDA 동시 분석법 설정 및 세포독성

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Simultaneous Determination of Seven Compounds by HPLC-PDA and Cytotoxicity of Samchulkunbi-tang

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ABSTRACT

Objectives : To develop and validate HPLC-PDA methods for simultaneous determination of seven constituents in Samchulkunbi-tang (SKT). Additionally, we investigated the cytotoxicity against BEAS-2B cell line and splenocytes of SKT.

Methods : Reverse-phase chromatography using a Gemini C_{18} column operating at 40° C, and photodiode array (PDA) detection at 230, 254 and 280 nm, were used for quantification of the three marker components of SKT. The mobile phase using a gradient flow consisted of two solvent systems. Solvent A was 1.0% (v/v) aqueous acetic acid and solvent B was acetonitrile with 1.0% (v/v) acetic acid. The cytotoxicity of SKT were measured by the CCK-8 assay method.

Results : Calibration curves were acquired with r^2〉0.9999, and the relative standard deviation (RSD) values (%) for intra- and inter-day precision were less than 6.0%. The recovery rate of each compound was in the range of 86.89-109.78%, with an RSD less than 4.0%. The contents of seven compounds in SKT were 1.39-6.84 mg/g. SKT had no cytotoxicity effect at 50-200 μg/mL concentrations.

Conclusions : The established method will be helpful to improve quality control and *in vitro* efficacy study of SKT.

Key words : HPLC-PAD, samchulkunbi-tang (SKT), simultaneous determination, cytotoxicity, BEAS-2B cell

Introduction

Many traditional herbal medicines have been used for prevention and therapy for diseases. Such medicines generally have few side effects and are very effective. Thus, traditional herbal medicines remain of considerable interest[1-3].

Samchulkunbi-tang (SKT, *shen zhu jian pi-tang* in Chinese) is a traditional herbal medicine widely used for the treatment of chronic gastritis, gastric ulcers, gastroptosis, indigestion, diarrhea, and emesis[4]. A composite traditional Korean medicine, SKT is a basic prescription of 14 herbs (Table 1). To date, pharmacological studies of SKT have been confined to its roles in gastroprotection and immune regulation[5].

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Table 1. Composition of Samchulkunbi-tang.

Latin name	Amount(g)	Company of purchase	Source	Marker substance[a)]
Ginseng Radix	3.75	Omniherb	Geumsan, Korea	Ginsenoside Rb1, Ginsenoside Rg1
Atractylodis Rhizoma	3.75	Omniherb	China	
Hoelen	3.75	Omniherb	Yeongcheon, Korea	
Magnoliae Cortex	3.75	HMAX	China	Magnolol
Citri Pericarpium	3.75	Omniherb	Jeju, Korea	Hesperidin
Crataegi Fructus	3.75	Omniherb	Inje, Korea	
Ponciri Fructus	3.00	HMAX	China	Poncirin
Paeoniae Radix	3.00	Omniherb	Hwasun, Korea	Albiflorin, Paeoniflorin
Amomi Semen	1.875	HMAX	China	
Massa Medicata Fermentata	1.875	Omniherb	Muju, Korea	
Hordei Fructus Germinatus	1.875	Omniherb	Muju, Korea	
Glycyrrhizae Radix	1.875	HMAX	China	Liquiritin, Glycyrrhizin
Zingiberis Rhizoma	3.75	Omniherb	Yeongcheon, Korea	6-Gingerol
Zizyphi Fructus	3.75	Omniherb	Yeongcheon, Korea	
Total	43.5			

a) Marker substances are based on 『The Korean Pharmacopoeia (KP, Ninth Edition)』.

Hesperidin and glycyrrhizin, constituents of SKT, have anti-oxidant[6], anti-inflammatory[7,8], and immunomodulatory effects[9,10]. Liquiritin has been shown to possess several pharmacological actions, including neuroprotective and anti-depressant activities[11,12]. Albiflorin and paeoniflorin, the major active constituents of Paeoniae Radix, are characteristic monoterpene glucosides isolated from the roots of *Paeonia lactiflora*[13,14]. In addition, paeoniflorin has been reported to exhibit anti-coagulant[15], neuromuscular blocking[16], immunoregulatory[17], and anti-hyperglycemic effects[18]. Additionally, poncirin and naringin, the major active compounds of Ponciri Fructus, have been found to have anti-*Helicobacter pylori*[19], anti-inflammatory[20], and anti-atherogenic effects[21]. Thus, the pharmacology of the preparation has received considerable attention. However, no study has yet used high-performance liquid chromatography (HPLC) to analyze SKT components. In addition, herbal decoctions in water are some of the most commonly consumed beverages in the world. In traditional Korean medicine, SKT has long been one of the most frequently prescribed herbal formulas for treating gastroptosis, indigestion, diarrhea, and emesis[4]. Therefore, to examine the cytotoxic potential of SKT, its effect on viability of BEAS-2B cell line and splenocytes were measured.

HPLC, coupled with other techniques, particularly photodiode array (PDA) detection, is a convenient, widely used, and powerful approach for rapid identification of constituents in botanical extracts and plants important in traditional Chinese medicine[22]. Therefore, we here focused on quantitative determination of the main components of SKT, and used HPLC-PDA-coupled methods for simultaneous detection of 7 constituents (Fig.1). Furthermore, we investigated the cytotoxicity against BEAS-2B cell line and splenocytes of SKT.

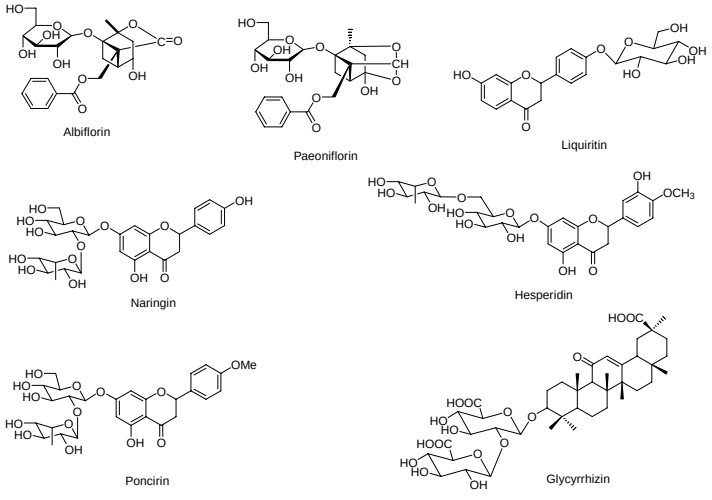

Figure 1. Chemical structures of 7 constituents in Samchulkunbi-tang.

Materials and methods

1. Chromatographic system

Analysis was performed using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan), consisting of a solvent delivery unit, an on-line degasser, a column oven, an autosampler, and a PDA detector. The data processor employed LCsolution software (Version 1.24). The analytical column used was a Gemini C_{18} (250×4.6 mm; particle size 5 μm; Phenomenex, Torrance, CA). The mobile phases were solvent A (1.0%, v/v, aqueous acetic acid) and solvent B (acetonitrile with 1.0%, v/v, acetic acid). The gradient flow was as follows: (A)/(B)=85/15 (0 min) → (A)/(B)=35/65 (35 min) → (A)/(B)= 0/100 (45 min; hold for 5 min) → (A)/(B)= 85/15 (55 min). Column temperature was maintained at 40° C. The analysis was carried out at a flow rate of 1.0 mL/min with PDA detection at 230, 254 and 280 nm. The injection volume was 10 μL.

2. Reagents and materials

Paeoniflorin, liquiritin, naringin, glycyrrhizin (all of purity ≥98.0%), and hesperidin (purity ≥92.0%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Albiflorin and poncirin (both of purity ≥98.0%) were obtained from ChromaDex (Irvine, CA, USA). HPLC-grade methanol, acetonitrile, and water were purchased from J.T. Baker (Phillipsburg, NJ). Glacial acetic acid was analytical reagent grade, procured from Merck KGaA (Darmstadt, Germany). The materials forming SKT were purchased from Omniherb (Yeongcheon, Korea) and HMAX (Chungbuk, Korea). All SKT components were taxonomically confirmed by Professor Je-Hyun Lee, Dongguk University, Gyeongju, Republic of Korea. Each voucher specimen (2008-KE06-1~KE06-14) has been deposited at the Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine.

3. Preparation of standard solutions and calibration curves

Standard stock solutions of 7 compounds (all at 1,000 μg/mL) were prepared in methanol and stored below 4° C. Working standard solutions were prepared by serial dilution of stock solutions with methanol. All calibration curves were obtained from assessment of peak areas of standard solutions in the concentration ranges: albiflorin, 0.78-50.00 μg/mL; paeoniflorin, 0.78-100.00 μg/mL; liquiritin, 0.39-50.00 μg/mL; naringin, 0.78-100.00 μg/mL; hesperidin, 0.78-100.00 μg/mL; poncirin, 0.78-100.00 μg/mL; and glycyrrhizin, 0.78-50.00 μg/mL.

4. Preparation of sample solutions

A decoction of SKT was prepared in our laboratory from a mixture of chopped crude herbs purchased from Omniherb (Yeongcheon, Korea) and HMAX (Chungbuk, Korea). SKT was prepared as described in Table 1 and extracted in distilled water at 100° C for 2 h. The extract was evaporated to dryness and freeze-dried (yield; 15.9%). Lyophilized SKT extract was weighed (200 mg) into a 20 mL flask and distilled water added to the volumetric mark. The mixture was shaken for 10 min at room temperature. After extraction, the mixture was passed through a 0.2 μm syringe filter, and 10 μL aliquots of filtrate were injected into the HPLC.

5. Limits of detection (LODs) and quantification (LOQs)

Stock solutions of reference compounds were further diluted with methanol to assess LOD and LOQ values. The LOD and LOQ data obtained under the chromatographic conditions used in this report were determined using signal-to-noise (S/N) ratios of 3 and 10, respectively.

6. Precision and accuracy

Repeatability was assessed by analysis of seven independently prepared standard solutions. The relative standard deviation (RSD) of peak areas of analytes, and peak retention times for each standard, were calculated.

Intra- and inter-day precision was determined using a standard addition method to prepare spiked samples, employing both standards and controls.

Recovery tests were performed by adding known amounts of reference standards to SKT samples before extraction. An average recovery was calculated using the formula : Recovery (%) = $(Amount_{determined} - Amount_{original})/Amount_{spiked} \times 100$.

7. Cell culture

The BEAS-2B cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine (1 mM), 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (50 U/mL) and streptomycin (50 μg/mL) at 37° C in an atmosphere of 5% (v/v) CO_2. Cells attaining a concentration of 3×10^3 cells/mL were activated by incubation in medium containing

Escherichia coli LPS (1 μg/mL). LPS was added to varying concentrations of test compounds dissolved in PBS. Cells treated with PBS were used as the vehicle control.

8. Cell viability

Cells were seeded into 96-well plates at a density of 3×10^3 cells/well, and incubated in serum-free medium in the presence of different concentrations of ADEE (25, 50, 100, and 200 μg/mL) for 24 h. The cell counting kit-8 (CCK-8) reagent (Dojindo, Japan) was added at 4 h. Thereafter, absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Benchmark; Bio-Rad Laboratories, CA, USA). Cell viability relative to that of the untreated control group was calculated. All experiments were performed in triplicate.

9. Preparation and treatment of splenocytes suspensions

Spleens from BALB/c mice were aseptically removed and a single-cell suspension obtained by passing the cells twice through a needle in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 25 mM HEPES, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (GibcoBRL, NY, USA). Red blood cells (RBC) were lysed with lysis buffer (Sigma Chemical Co., St Louis, MO, USA) at 37° C for 10 min. After washing with PBS, cells were cultured in 100f dishes for 4 h. Splenocytes were plated onto 96-well plates at a density of 1×10^6 cells/mL and treated with different concentrations of *p*-hydroxycinnamic acid methylester for 1 h, followed by treatment with ConA (1 μg/mL) for a further 3 days. All experimental procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of Korea.

10. Statistical analysis

Results are presented as means ± standard error (SEM) of three samples and are representative of two independent experiments. Statistical significance was determined using a two-tailed Student's t-test for independent means.

Results

1. Optimization of chromatographic conditions

We obtained satisfactory separation using mobile phases consisting of (A) 1.0% (v/v) aqueous acetic acid and (B) acetonitrile with 1.0% (v/v) acetic acid, with a gradient flow of (A)/(B)=85/15 (0 min) → (A)/(B)=35/65 (35 min) → (A)/(B)= 0/100 (45 min, with a hold for 5 min) → (A)/(B)= 85/15 (55 min). Quantitation was achieved by PDA detection from 190-400 nm, based on peak area. The selectivity of the HPLC protocol is illustrated in Fig. 2, where good separation of marker compounds from other components of the extract can be noted. Using optimized chromatography conditions, all analytes were eluted before 35 min, showing a resolution better than 0.7, and good specificity in sample analysis. Representative HPLC chromatograms of standards and extract are shown in Fig. 2.

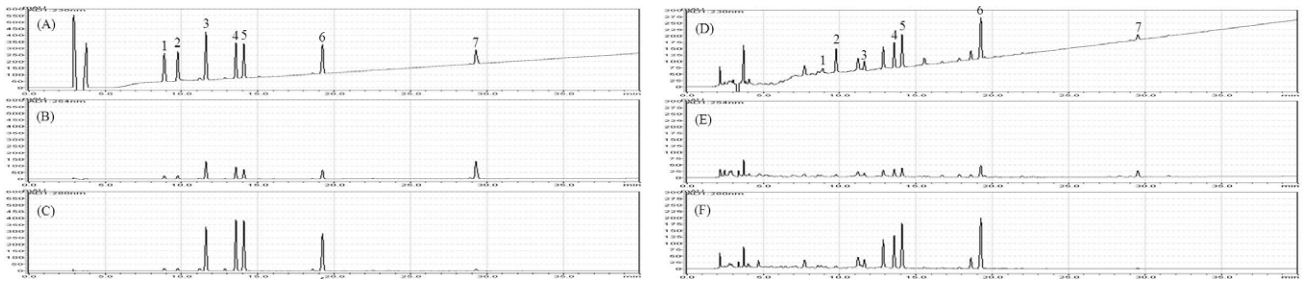

Figure 2. HPLC chromatogram of a standard mixture of 7 major compounds with detection at 230 nm (A), 254 nm (B), and 280 nm (C). Components of a Samchulkunbi-tang extract detected at 230 nm (D), 254 nm (E), and 280 nm (F). Albiflorin (1), paeoniflorin (2), liquiritin (3), naringin (4), hesperidin (5), poncirin (6), and glycyrrhizin (7).

2. Recovery

A recovery test was performed by addition of known amounts of albiflorin, paeoniflorin, liquiritin, naringin, hesperidin, poncirin, and glycyrrhizin. Standard compounds, at each of two different levels, were mixed with sample powder, and extracted. The recovery of each standard ranged from 86.89-109.78%, and the RSD range was 0.15-3.79% (Table 2).

Table 2. Recovery of seven marker compounds (n=3).

Compound	Original mean(ug/mL)	Spiked(ug/mL)	Detected mean(ug/mL)	Recovery mean(%)	SD	RSD (%)
Albiflorin	11.73	2.00	13.74	100.39	3.27	3.26
		5.00	16.08	86.89	3.30	3.79
Paeoniflorin	53.29	10.00	63.82	105.30	2.97	2.82
		25.00	78.57	101.12	1.19	1.17
Liquiritin	17.73	4.00	21.58	96.10	1.59	1.65
		10.00	27.26	95.33	0.88	0.92
Naringin	52.45	10.00	61.94	94.95	0.27	0.29
		25.00	75.93	93.92	0.14	0.15
Hesperidin	70.24	10.00	79.97	97.28	0.75	0.77
		25.00	94.44	96.82	0.28	0.29
Poncirin	58.34	5.00	63.83	109.78	1.53	1.39
		12.50	71.81	107.77	0.57	0.53
Glycyrrhizin	28.95	6.00	34.65	94.92	0.16	0.17
		15.00	42.64	91.26	0.13	0.15

3. Linearity, range, LOD, and LOQ

Calibration curves were obtained using standard solutions containing 0.78-50.00 μg/mL of albiflorin, 0.78-100.00 μg/mL of paeoniflorin, 0.39-50.00 μg/mL of liquiritin, 0.78-100.00 μg/mL of naringin, 0.78-100.00 μg/mL of hesperidin, 0.78-100.00 μg/mL of poncirin, and 0.39-50.00 μg/mL of glycyrrhizin as marker compounds. Line equations representing calibration curves and correlation coefficients thereof are summarized in Table 3.

The ranges of LODs and LOQs were 0.05-0.23 μg/mL and 0.15-0.78 μg/mL, respectively. These data are summarized in Table 3.

Table 3. Regression data, linear ranges, LODs and LOQs for marker compounds.

Compound	Linear range(ug/mL)	Slope	Intercept	Correlation coefficient(R^2)	LOD(ug/mL)	LOQ(ug/mL)
Albiflorin	0.78 - 50.00	11938.0	-45.6	1.0000	0.13	0.45
Paeoniflorin	0.78 - 100.00	12182.0	-3175.6	0.9999	0.16	0.52
Liquiritin	0.39 - 50.00	17784.0	-118.0	1.0000	0.07	0.24
Naringin	0.78 - 100.00	17210.0	-470.6	1.0000	0.06	0.18
Hesperidin	0.78 - 100.00	18422.0	-534.2	1.0000	0.06	0.19
Poncirin	0.78 - 100.00	28646.0	-751.8	1.0000	0.05	0.15
Glycyrrhizin	0.78 - 50.00	8246.9	-345.0	1.0000	0.20	0.65

4. Accuracy and precision

Repeatability or intra-assay precision was assessed by repeatedly measuring retention times and peak areas for three independently prepared samples of analytes. Measurement precision was less than RSD 1.5% for peak responses and less than RSD 0.1% for retention times, with all analytes. Thus, the HPLC assay offered good reproducibility under optimized conditions.

To test the accuracy and precision of our analytical method, intra- and inter-day variations for measurement of seven marker compounds were determined, and are summarized in Table 4. The precision of the method in simultaneous determination of the three marker compounds was acceptable because the RSD did not exceed 6.0% at any concentration. The intra-day accuracy ranged from 98.40-102.47%, and the inter-day accuracy from 98.34-110.36%.

Table 4. Precision and accuracy of analytical results (n=3).

Compound	Spiked Conc.(ug/mL)	Intra-day			Inter-day		
		Observed Conc. (ug/mL)	Precision (RSD %)	Accuracy (%)	Observed Conc. (ug/mL)	Precision (RSD %)	Accuracy (%)
Albiflorin	2.00	2.05	5.11	102.47	2.21	2.94	110.36
	5.00	4.98	0.84	99.61	4.92	0.53	98.34
Paeoniflorin	10.00	9.76	0.43	97.59	10.27	1.72	102.73
	25.00	25.10	0.07	100.39	24.89	0.28	99.56
Liquiritin	4.00	4.05	2.12	101.22	4.02	1.13	100.53
	10.00	9.98	0.34	99.81	9.99	0.18	99.91
Naringin	10.00	9.91	0.91	99.09	10.07	0.23	100.72
	25.00	25.04	0.14	100.15	24.97	0.04	99.88
Hesperidin	10.00	9.90	1.34	98.98	10.03	0.53	100.32
	25.00	25.04	0.21	100.16	24.99	0.08	99.95
Poncirin	5.00	4.92	1.79	98.39	5.06	0.65	101.23
	12.50	12.53	0.28	100.26	12.48	0.10	99.80
Glycyrrhizin	6.00	6.03	0.98	100.54	6.16	0.10	102.64
	15.00	14.99	0.16	99.91	14.94	0.02	99.58

5. Determination of the main constituents of SKT

Our assay was subsequently applied to the simultaneous determination of 7 compounds, albiflorin, paeoniflorin, liquiritin, naringin, hesperidin, poncirin and glycyrrhizin, in SKT. Fig. 2 show chromatograms of reference compounds and that of a water extract of SKT, with detection of eluents at 230 nm, 254 nm, and 280 nm. The analytical results for each compound identified are summarized in Table 5.

Table 5. Analytical results for marker compounds in Samchulkunbi-tang.

Batch (#)	Compound											
	Albiflorin			Paeoniflorin			Liquiritin			Naringin		
	Mean(mg/g)	SD	RSD(%)	Mean(mg/g)	SD	RSD(%)	Mean(mg/g)	SD	RSD(%)	Mean(mg/g)	SD	RSD(%)
1	1.39	0.009	0.637	5.55	0.057	1.021	1.61	0.004	0.269	4.98	0.000	0.007
2	1.41	0.008	0.576	5.64	0.011	0.191	1.60	0.014	0.850	5.02	0.009	0.181
3	1.39	0.009	0.613	5.60	0.003	0.048	1.60	0.006	0.375	5.00	0.016	0.327

Batch (#)	Compound								
	Hesperidin			Poncirin			Glycyrrhizin		
	Mean(mg/g)	SD	RSD(%)	Mean(mg/g)	SD	RSD(%)	Mean(mg/g)	SD	RSD(%)
1	6.76	0.009	0.129	5.44	0.009	0.170	2.78	0.008	0.290
2	6.84	0.002	0.034	5.47	0.004	0.076	2.81	0.004	0.127
3	6.79	0.010	0.143	5.46	0.008	0.154	2.79	0.005	0.170

6. Cytotoxicity

To determine cell viability, BEAS-2B and splenocytes were incubated with different concentrations of SKT extract (50, 100 and 200 μg/mL) for 24 h. As shown in Fig. 3, up to 200 μg/mL. SKT did not influence cell viability in cultured BEAS-2B and splenocytes, and influenced cell viability only slightly at a concentration of 200 μg/mL of splenocytes.

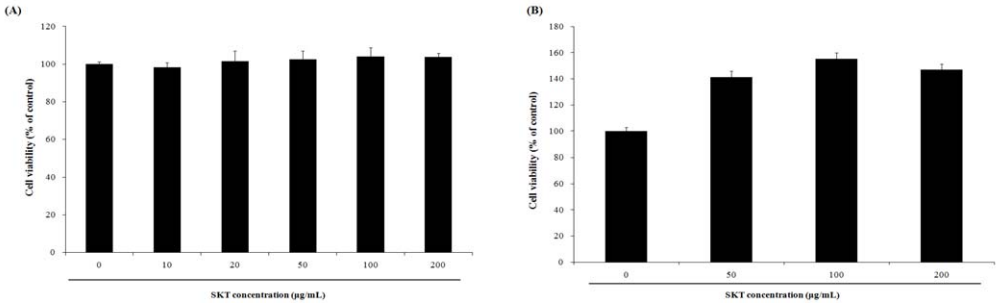

Figure 3. Effect of the Samchulkunbi-tang extract on cell viability. BEAS-2B cell (A) and splenocyte (B).

Discussion

This study described a simultaneous determination for the qualification and quantitation of seven compounds of SKT. It consist of 14 herbs including Ginseng Radix, Atractylodis Rhizoma, Hoelen, Magnoliae Cortex, Citri Pericarpium, Crataegi Fructus, Ponciri Fructus, Paeoniae Radix, Amomi Semen, Massa Medicata Fermentata, Hordei Fructus Germinatus, Glycyrrhizae Radix, Zingiberis Rhizoma, and Zizyphi Fructus. In those herbal medicines, marker constituents on 7 herbal medicines such as Ginseng Radix, Magnoliae Cortex, Citri Pericarpium, Ponciri Fructus, Paeoniae Radix, Glycyrrhizae Radix, and Zingiberis Rhizoma are included in the Korean Pharmacopoeia (KP, Ninth Edition). Therefore, we try to analyze seven compounds including hesperidin, naringin, poncirin, albiflorin, paeoniflorin, liquiritin, and glycyrrhizin among 7 herbal medicines. Hesperidin, naringin, and poncirin which are major compounds of Citri Pericarpium and Ponciri Fructus, respectively have anti-oxidant[6], anti-inflammatory[7], and immunomodulatory[10], anti-*Helicobacter pylori*[19], anti-inflammatory[20], and anti-atherogenic effects[21]. Albiflorin and paeoniflorin which are major constituents of Paeoniae Radix exhibited anti-coagulant[15], neuromuscular blocking[16], immunoregulatory[17], and anti-hyperglycemic effects[18]. Additionally, glycyrrhizin and liquiritin, major constituents of Glycyrrhizae Radix, have anti-inflammatory[8], immunomodulatory[9], anti-depressant[11], and neuroprotective activities[12]. By optimizing HPLC condition, seven compounds of SKT could be determined by HPLC within 35 min using two solvent system containing 1.0% acetic acid at 40° C. In the present work, simultaneous determination of the seven marker compounds in SKT was validated with respect to linearity, precision, and accuracy. The method will help to improve quality control of SKT. Additionally, in the evaluation of the *in vitro* cytotoxicity of SKT

against the BEAS-2B cell lines and splenocytes, had no cytotoxic effects upto 200 ug/mL. Consequently, SKT would clearly have beneficial uses in the biological acitivity assay.

Acknowledgements

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