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SIMULTANEOUS DETERMINATION AND ANTI-INFLAMMATORY EFFECTS OF TRADITIONAL HERBAL MEDICINE, MAHWANG-TANG

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Abstract

Background: Mahwang-tang (MT) is a traditional Korean medicine consisting of six medicinal herbs and is used to treat the influenza-like diseases.

Materials and Methods: We performed the simultaneous analysis of nine bioactive components in an MT sample using a high-performance liquid chromatography–diode array detector (HPLC–DAD) analysis. In addition, the MT sample was investigated the inhibitory effects against lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

Results: Recovery of the nine marker compounds was 98.24–102.05% and relative standard deviations of intra-day and inter-day precisions of this method were 0.05–1.92% and 0.02–1.64%, respectively. Amounts of the nine bioactive compounds in the MT samples were 0.24–24.86 mg/g. To determine the biological activity of MT, its effects on the inflammatory reaction in LPS-treated RAW 264.7 macrophages were tested. The results suggest that MT possesses anti-inflammatory activity via suppression of the nitric oxide/prostaglandin E_2 pathway.

Conclusion: The established analytical method by HPLC–DAD is expected to help the quality control of MT samples or related herbal prescriptions. Our data also suggest that MT may be a potential therapeutic candidate for various inflammatory diseases.

Key words: Simultaneous determination; anti-Inflammatory effect; traditional herbal medicine; Mahwang-tang, HPLC–PDA

Introduction

Traditional herbal formulations commonly combine two or more medicinal herbs. Therefore, they contain many components and exhibit various biological activities. One of these traditional Korean herbal formulas, Mahwang-tang (MT) was first recorded in *Shanghan Lun* by Zhang Zhongjing in the later years of the Han dynasty (AD 200). Since then, it has been recorded in *Dongui Bogam* by Heo Jun in Korea (AD 1613). The MT decoction is manufactured from six herbs, Ephedrae Herba, Cinnamomi Ramulus, Glycyrrhizae Radix et Rhizoma, Armeniacae Semen, Zingiberis Rhizoma Crudus, and Allii Radix, combined in the ratio 5:3.3:1:1.7:1.7:1.7 based on dry weight. It has been used for many years in Korea to treat influenza-like symptoms such as headache, high fever, cough, and arthralgia (Heo, 2004). MT has been reported to show antipyretic (Kubo and Nishimura, 2007), viral myocarditic (Shijie et al., 2010), anti-asthma (Ma et al., 2014), and chronic hepatitis C (Kainuma et al., 2002a; Kainuma et al., 2002b) effects. Analytical methods to determine various components—ephedrine, amygdalin, cinnamic acid, cinnamaldehyde, liquiritin, and glycyrrhizin—in a decoction of MT or Kampo medicines containing Ephedrae Herba using a reversed-phase high-performance liquid chromatography (RP–HPLC) coupled with a UV–Vis or diode array detector (DAD) method have been reported (Okamura et al., 1999a; Okamura et al., 1999b; He et al., 2012). However, these methods were only quantitative analyses of the main components in MT or Kampo medicines containing Ephedrae Herba. In addition, there is a limit to verifying the analytical method.

Inflammation is the body's protective response against injurious stimuli, including bacteria, damaged cells, or irritants (Ferrero-Miliani et al., 2007). Various types of immune cells are involved in regulation of the immune response. In particular, macrophages display anti-inflammatory action and reduce the immune reaction through the production of pro-inflammatory mediators, including nitric oxide (NO) and prostaglandin E_2 (PGE_2) (Fujiwara and Kobayashi, 2005; Mills, 2012). Macrophage stimulation with immune stimulants such as lipopolysaccharide (LPS) mediates inflammation by enhancing the production of pro-inflammatory mediators (MacMicking et al., 1997). In this study, we examined the inhibitory effect on the inflammation of MT using RAW 264.7 murine macrophages. The inhibition of NO and PGE_2 production, and expression of inducible nitric oxide (iNOS) and cyclooxygenase-2 (COX-2) by MT was evaluated to determine its anti-inflammatory activity in LPS-stimulated RAW 264.7 cells.

In this study, HPLC analysis for simultaneous quantification of the nine components, that is, ephedrine HCl (**1**), amygdalin (**2**), liquiritin apioside (**3**), liquiritin (**4**), coumarin (**5**), cinnamic acid (**6**), cinnamaldehyde (**7**), glycyrrhizin (**8**), and 6-gingerol (**9**) in the MT sample was conducted using HPLC–DAD. In addition, we also evaluated the anti-inflammatory activity of the MT decoction using RAW 264.7 cell line.

Materials and Methods

Plant materials

Six raw herbs (Table 1), Ephedrae Herba, Cinnamomi Ramulus, Glycyrrhizae Radix et Rhizoma, Armeniacae Semen, Zingiberis Rhizoma Crudus, and Allii Radix were provided from the Korean herbal market, Naemome Dah (Ulsan, Korea), in February 2012. The botanical origins of the six crude materials were confirmed by pharmacognosists, Professor Je-Hyun Lee, College of Oriental Medicine, Dongguk University (Gyeongju, Korea) and Young-Bae Seo, College of Oriental Medicine, Daejeon University (Daejeon, Korea) according to the guidelines on the visual and

organoleptic examination of herbal medicine (Moon, 2006; Yun, 2008; Kim, 2011). Specimens of raw materials (2012–KE48–1 to KE48–4) have been stored at the K-herb Research Center, Korea Institute of Oriental Medicine (KIOM).

Table 1: Single dose composition of MT.

Herbal medicine	Scientific name	Family	Origin	Amount (g)
Ephedrae Herba	*Ephedra sinica* Stapf	Ephedraceae	China	11.25
Cinnamomi Ramulus	*Cinnamomum cassia* Presl	Lauraceae	Vietnam	7.50
Glycyrrhizae Radix et Rhizoma	*Glycyrrhiza uralensis* Fischer	Leguminosae	China	2.25
Armeniacae Semen	*Prunus armeniaca* Linne var. ansu Maximowicz	Rosaceae	China	3.75
Zingiberis Rhizoma Crudus	*Zingiber officinale* Roscoe	Zingiberaceae	Ulsan, Korea	3.75
Allii Radix	*Allium fistulosum* Linne	Liliaceae	Hanam, Korea	3.75
Total amount				32.25

Chemicals and reagents

For the reference standards, compound **1** (95.0%) was provided by the Ministry of Food and Drug Safety (Cheongju, Korea); compounds **2** and **5** (both 99.0%) from Merck KGaA (Darmstadt, Germany); compound **3** (98.0%) from Shanghai Sunny Biotech (Shanghai, China); and compounds **4** (99.6%), **6** (98.0%), **7** (98.0%), **8** (99.0%), and **9** (99.0%) from Wako Chemicals (Osaka, Japan). The chemical structures of the nine biomarker components subjected to quantitative analysis are shown in Fig. 1. The methanol, acetonitrile, and water used in the study were all HPLC-grade products and obtained from J.T. Baker (Phillipsburg, NJ, USA). The 2,2,2-trifluoroethanoic acid (TFA) for HPLC (≥99.0%) was provided from Merck KGaA (Darmstadt, Germany).

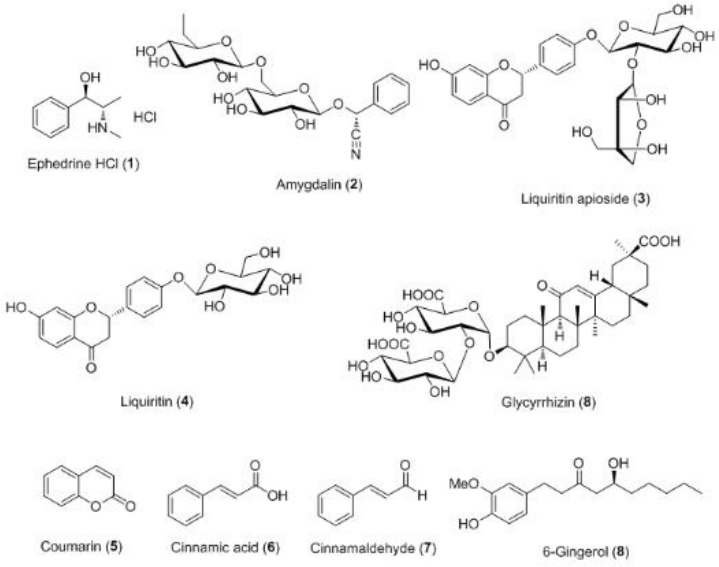

Figure 1: Chemical structure of the nine bioactive compounds in MT.

Preparation of standard solutions

Individual standard stock solutions of the nine reference standards were prepared at a concentration of 1000 μg/mL using methanol and stored at 4°C until use.

Preparation of the MT water extract and quality control sample

MT water extract was composed of six crude herbal medicines as listed in Table 1 and prepared in KIOM. The six raw materials, 1,744 g of Ephedrae Herba, 1,163 g of Cinnamomi Ramulus, 349 g of Glycyrrhizae Radix et Rhizoma, 581 g of Armeniacae Semen, 581 g of Zingiberis Rhizoma Crudus, and 581 g of Allii Radix were mixed and placed in a 10-times mass of distilled water (50 L), then boiled for 2 h at 100°C under a pressure of 0.98 bar using an electric extractor (COSMOS-660; Kyungseo Machine Co., Incheon, Korea). The extracted water solution was filtered via the a standard sieve (No. 270, 53 μm, 203 ∅; Chung Gye Sang Gong Sa, Seoul, Korea); then the filtered samples were lyophilized using a freezing dryer, PVT100 (IlShinBioBase, Yangju, Korea) to obtain a plwdered MT sample. The amount of lyophilized MT powder obtained was 226.2 g (yield: 4.5%). For the quantitative analysis of the nine biomarker compounds using HPLC–DAD, 100.0 mg of the freeze-dried MT sample was dissolved in 20 mL of 70% methanol by sonication for 20 min; then, the prepared sample solution for analysis of compounds **1**, **2**, and **7** was diluted twofold and filtered via a 0.2 μm membrane filter (PALL Life Sciences, Ann Arbor, MI, USA) prior to injection to the HPLC equipment.

Chromatographic analysis of the MT sample

Chromatographic analysis was carried out on a Shimadzu Prominence LC-20A series HPLC system (Kyoto, Japan) equipped with a Model LC-20AT solvent delivery pump, SIL-20A autosampler, CTO-20A column oven, DGU-20A3 degasser, SPD-M20A DAD, and Lab Solutions software (Version 5.54 SP3). Waters SunFire C_{18} reversed-phased column (4.6 × 250 mm; 5 μm, Milford, MA, USA) was used for the separation of the nine bioactive components and retained at 40°C. The mobile phases consisted of 0.1% (v/v) TFA in distilled water (A) and acetonitrile (B) and the gradient system for chromatographic separation was as follows: 10–60% B for 0–30 min, 60–100% B for 30–40 min, 100% B for 40–45 min, 100–10% B for 45–50 min, and 10% B for 50–60 min with a flow rate of 1.0 mL/min. Injection volume was 10 μL.

Calibration curves, limits of detection (LOD), and limits of quantification (LOQ)

Calibration curves were draw up by plotting the peak areas (y) of each compound against their corresponding concentration (x, μg/mL) using prepared standard solutions. Samples were measured in triplicate to obtain a regression equation including slope, intercept, and correlation coefficient. The LOD and LOQ values were calculated using the calibration curve of each reference standard as follows: LOD = $3.3\sigma \times S$ and LOQ = $10\sigma \times S$, where σ is the standard deviation of the blank response and S is the slope of the calibration curve.

Precision and accuracy

To determine the precision of the established HPLC–DAD method, intra- and inter-day tests were carried out within one day and three consecutive days according to the International Conference on Harmonization (ICH) guideline (ICH, 2005). Intra-day and inter-day precisions were assessed using the relative standard deviation (RSD) value as an evaluation index and RSD (%) was calculated by the following equation: RSD (%) = 100 × standard deviation (SD)/mean. Reproducibility of this method was evaluated by the RSD value of the amount and retention time of each reference standard. The recovery was performed to assess the accuracy of this analytical method using the standard addition method and the recovery of each compound was calculated using the following equation: recovery (%) = 100 × (found amount – original amount)/spiked amount.

Cell culture

Mouse macrophages RAW 264.7 (American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Inc., Grand Island, NY, USA) containing 5.5% heat-inactivated fetal bovine serum (Gibco Inc.), penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively, Thermo Fisher Scientific, Rockford, IL, USA) at 37°C.

Cytotoxicity assay

RAW 264.7 cells were exposed to MT extract for 24 h. The cytotoxic effect of MT against RAW 264.7 macrophages was assessed using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). The cell viability was calculated using the following equation:

$$\text{Cell viability } (\%) = \frac{\text{Mean OD at 450 nm in MT-exposed cells}}{\text{Mean OD at 450 nm in control cells}} \times 100$$

Measurement of NO and PGE_2 production

Cell supernatants were collected from RAW 264.7 cells treated with LPS (1 μg/mL) in the presence or absence of MT extract, and subjected for analysis of NO generation (Griess Reagent System; Promega Corp., Madison, WI, USA) and PGE_2 production (ELISA kit; Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturers' instructions.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using Trizol reagent (Invitrogen Life Sciences, Carlsbad, CA, USA). cDNA was generated by the reverse transcription and subjected to PCR reactions using rTaq DNA polymerase (ELPIS Biotech

Inc., Daejeon, Korea). The relative mRNA levels of iNOS and COX-2 were analyzed and adjusted by the β-actin expression. The amplification primers listed in Table 2.

Table 2: List of primer sequences.

Primer	Sequences	Temperature
iNOS	Forward 5′- TCA CCT ACT TCC TGG ACA TTA -3′	59°C
	Reverse 5′- ACT TCC AGT CAT TGT ACT CTG -3′	
COX-2	Forward 5′-GTA TCA GAA CCG CAT TGC CTC TGA-3′	59°C
	Reverse 5′-CGG CTT CCA GTA TTG AGG AGA ACA GAT-3′	
β-actin	Forward 5′-ACC GTG AAA AGA TGA CCC AG-3′	57°C
	Reverse 5′-TAC GGA TGA CAA CGT CAC AC-3′	

Statistical analysis

All values are expressed as the mean ± S.E.M. from three independent assays. The significant differences were considered significant at $P < 0.05$.

Results and Discussion

Optimization of chromatographic conditions

To establish the optimal HPLC–DAD method for simultaneous analysis of the target analytes in the MT sample, the nine compounds shown in Fig. 1 were selected as marker compounds. These compounds belong to different chemical classes. Therefore, gradient elution was examined for simultaneous analysis of these compounds, such as a variety of acidic mobile phases (namely, acetic acid, formic acid, and trifluoroacetic acid) and organic solvents (namely, methanol and acetonitrile). Furthermore, HPLC parameters were also tested as follows: different C_{18} columns, namely a Phenomenex Gemini C_{18}, Waters SunFire C_{18}, and Shiseido Capcell Pak UG120 C_{18} (all 4.6 × 250 mm, 5 μm) and column temperatures (30, 35, and 40°C) to improve chromatographic separation with good baseline, resolution, and peak tailing. Thus, the optimal chromatographic separation conditions determined were a Waters SunFire C_{18} column at a column temperature of 40°C. In addition, the mobile phase was determined as a gradient flow of 0.1% (v/v) TFA in distilled water–acetonitrile systems. UV wavelengths for quantitative analysis of each analyte in the MT sample were set at 206 nm for compounds **1** and **2**, 254 nm for compound **8**, 275 nm for compounds **3**–**6**, 280 nm for compound **9**, and 288 nm for compound **7**.

System suitability

System suitability of the established HPLC–DAD analytical method was confirmed by the parameters, a capacity factor (k'), selectivity factor (α), resolution (Rs), theoretical plate number (N), and tailing factor (Tf). The values for these parameters are shown in Table 3.

Table 3: System suitability of the nine biomarker compounds.

Compound	Capacity factor (k')	Separation factor (α)	Number of theoretical plates (N)	Resolution (Rs)	Tailing factor (Tf)
1	2.36	1.14	9494	2.28	1.13
2	2.69	1.54	9704	2.28	0.91
3	4.14	1.54	24953	1.12	1.13
4	4.28	1.52	24830	1.12	1.09
5	6.51	1.52	22357	4.66	1.04
6	7.42	1.14	31709	4.66	1.05
7	8.42	1.03	39641	1.34	1.30
8	8.68	1.21	38610	1.34	1.19

Linearity, LOD, and LOQ

The linearity, which is expressed by the coefficient of determination (r^2) values of the nine compounds in the established analytical method ranged between 0.9999 and 1.0000. The LOD and LOQ values of all the analytes were 0.01–0.38 and 0.02–01.16 μg/mL, respectively. These results suggest that the linearity and sensitivity are very good in the seven tested concentration ranges for this analytical method (Table 4).

Table 4: Linear range, regression equation, r^2, LOD, and LOQ for the nine bioactive compounds in the MT sample.

Compound	Linear range (μg/mL)	Regression equation[a]	r^2	LOD[b] (μg/mL)	LOQ[c] (μg/mL)
1	7.81–125.00	$y = 21151.31x + 8837.74$	0.9999	0.16	0.50
2	7.81–500.00	$y = 9380.10x + 564.20$	0.9999	0.37	1.12
3	1.56–100.00	$y = 16795.26x – 4999.15$	1.0000	0.36	1.08
4	1.56–100.00	$y = 34977.41x – 5507.76$	1.0000	0.17	0.52
5	1.56–100.00	$y = 61766.38x – 21853.95$	0.9999	0.10	0.29
6	0.78–50.00	$y = 85141.39x – 16724.01$	0.9999	0.07	0.21
7	1.56–100.00	$y = 128109.55x – 28516.41$	1.0000	0.01	0.02
8	1.56–100.00	$y = 8700.95x – 1917.68$	1.0000	0.38	1.16
9	0.31–20.00	$y = 5605.95x + 1851.10$	0.9999	0.02	0.07

[a]y: peak area (mAU) of compounds; x: concentration (μg/mL) of compounds. [b]LOD = $3.3\sigma \times S$. [c]LOQ = $10\sigma \times S$.

Recovery and precision

The recovery was examined to assess the accuracy of the established analytical method in the MT sample. Extraction recoveries of compounds **1**–**9** in this method were 98.24–102.05% and the RSD was within 1.50% (Table 5).

RSD values for reproducibility evaluation were in the range 0.09–2.40% for the amount of the nine bioactive compounds and 0.03–0.28% for retention times of all the analytes (Table 6). In addition, the RSD values for intra-day and inter-day precision assays of the established analytical method were 0.05–1.92% and 0.02–1.64%, respectively (Table 7). These results suggest that the proposed analytical method for the quantification analysis of the nine bioactive components in the MT sample is a suitable method.

Table 5: Recovery of the nine compounds in MT.

Analyte	Original amount (μg/mL)	Spiked amount (μg/mL)	Found amount (μg/mL)	Recovery[a] (%)	RSD (%)
1	38.17	8.00	46.18	100.14	1.16
		20.00	57.86	98.67	0.45
		40.00	77.73	98.89	0.34
2	54.36	12.00	66.46	100.83	1.50
		30.00	84.92	101.88	0.29
		60.00	113.39	98.39	0.46
3	23.88	4.00	27.88	99.94	1.28
		10.00	33.87	99.89	0.98
		20.00	43.57	98.41	0.29
4	11.62	2.00	13.61	99.91	1.25
		5.00	16.61	99.92	0.78
		10.00	21.58	99.67	0.71
5	24.22	4.00	28.20	99.38	0.96
		10.00	34.24	100.20	0.50
		20.00	44.02	99.00	0.54
6	8.86	2.00	10.86	100.10	0.71
		5.00	13.84	99.53	0.24
		10.00	19.00	101.40	0.24
7	34.09	8.00	41.95	98.24	0.29
		15.00	49.40	102.05	0.36
		30.00	63.89	99.31	0.28
8	36.32	6.00	42.29	99.42	0.82
		15.00	51.10	98.50	0.50
		30.00	65.96	98.80	0.24
9	1.73	1.00	2.73	100.34	1.00
		2.00	3.74	100.35	0.33
		4.00	5.76	100.82	0.81

[a]Recovery (%) = 100 × (Found amount – Original amount)/Spiked amount.

Table 6: Reproducibility of retention times and amount for the nine bioactive components (n = 6).

Analyte	Retention time (min)		Amount (μg/mL)	
	Mean ± SD ($\times 10^{-1}$)	RSD (%)	Mean ± SD	RSD (%)
1	9.11 ± 0.06	0.07	38.17 ± 0.04	0.09
2	9.96 ± 0.04	0.04	54.32 ± 0.19	0.35
3	13.91 ± 0.23	0.16	24.04 ± 0.50	2.08
4	14.29 ± 0.40	0.28	11.65 ± 0.28	2.40
5	20.31 ± 0.23	0.12	24.23 ± 0.15	0.61
6	22.77 ± 0.22	0.10	8.86 ± 0.02	0.21
7	25.44 ± 0.09	0.03	34.07 ± 0.09	0.26
8	26.17 ± 0.22	0.08	36.31 ± 0.06	0.16
9	31.14 ± 0.23	0.07	1.73 ± 0.01	0.83

Table 7: Intra- and inter-day precision assays of the nine bioactive components in MT.

Compound	Spiked Conc. (μg/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
		Observed Conc. (μg/mL)	Precision (%)	Accuracy (%)	Observed Conc. (μg/mL)	Precision (%)	Accuracy (%)
1	8.00	8.09	0.62	101.08	8.07	0.71	100.84
	20.00	19.91	0.14	99.55	19.89	0.35	99.44
	40.00	40.03	0.05	100.07	40.04	0.08	100.11
2	12.00	11.96	0.90	99.68	11.99	0.94	99.93
	30.00	30.72	0.32	102.41	30.68	0.55	102.27
	60.00	59.65	0.07	99.41	59.66	0.14	99.44
3	4.00	4.01	0.47	100.20	4.04	0.94	101.06
	10.00	10.10	0.48	100.97	10.11	1.64	101.09
	20.00	19.95	0.13	99.75	19.94	0.40	99.68
4	2.00	2.00	1.14	100.02	2.00	0.88	100.01
	5.00	5.01	0.80	100.16	5.01	0.87	100.26
	10.00	10.00	0.17	99.96	9.99	0.21	99.93
5	4.00	3.98	0.54	99.59	3.96	0.80	98.98
	10.00	10.09	0.57	100.87	10.17	0.64	101.66
	20.00	19.96	0.13	99.80	19.93	0.13	99.66
6	2.00	2.00	0.47	100.17	2.01	0.46	100.41
	5.00	4.94	0.25	98.75	4.93	0.10	98.55
	10.00	10.03	0.05	100.31	10.03	0.02	100.35

	8.00	7.84	0.19	98.06	7.78	0.19	97.29
7	15.00	15.33	0.36	102.18	15.30	0.10	102.00
	30.00	29.88	0.10	99.59	29.91	0.02	99.69
	6.00	6.08	1.28	101.31	5.98	1.10	99.72
8	15.00	15.07	1.92	100.44	15.16	1.52	101.04
	30.00	30.23	1.56	100.77	29.93	0.36	99.75
	1.00	1.00	0.62	99.89	0.99	1.34	99.24
9	2.00	1.99	0.70	99.71	2.01	0.79	100.37
	4.00	4.00	0.14	100.08	4.00	0.20	99.95

HPLC analysis of the nine bioactive components in the MT sample

The HPLC–DAD analytical method established here was successfully applied to analyze compounds **1**–**9** in the MT sample simultaneously. All target compounds were identified based on a comparison of the retention time and UV spectra of each reference standard. Thus, the compounds **1**–**9** were detected at 9.11, 9.96, 13.91, 14.29, 20.31, 22.77, 25.44, 26.17, and 31.14 min, respectively (Fig. 2). Contents of the nine bioactive compounds in freeze-dried MT samples were in the range of 0.24–24.86 mg/g (Table 8). The results showed that the amount of most components determined is similar in different batches. In addition, the determined nine components could be considered as quality assessment markers for consistent research on MT quality.

Table 8: Contents of the nine bioactive compounds in the MT sample.

Analyte	Content (mg/g) ± SD ($\times 10^{-2}$) of lyophilized sample (n = 3)		
	Sample 1	Sample 2	Sample 3
1	15.47 ± 1.03	15.60 ± 3.31	15.32 ± 1.45
2	24.65 ± 8.56	24.86 ± 2.21	24.07 ± 43.96
3	5.22 ± 0.01	5.26 ± 0.54	5.14 ± 6.47
4	2.32 ± 1.17	2.36 ± 1.39	2.31 ± 0.03
5	4.97 ± 0.32	5.03 ± 0.64	4.93 ± 1.97
6	1.86 ± 0.14	1.87 ± 0.14	1.82 ± 3.13
7	13.80 ± 5.05	13.87 ± 7.71	13.80 ± 9.28
8	7.36 ± 0.91	7.44 ± 0.78	7.33 ± 0.32
9	0.25 ± 0.37	0.24 ± 0.45	0.25 ± 0.45

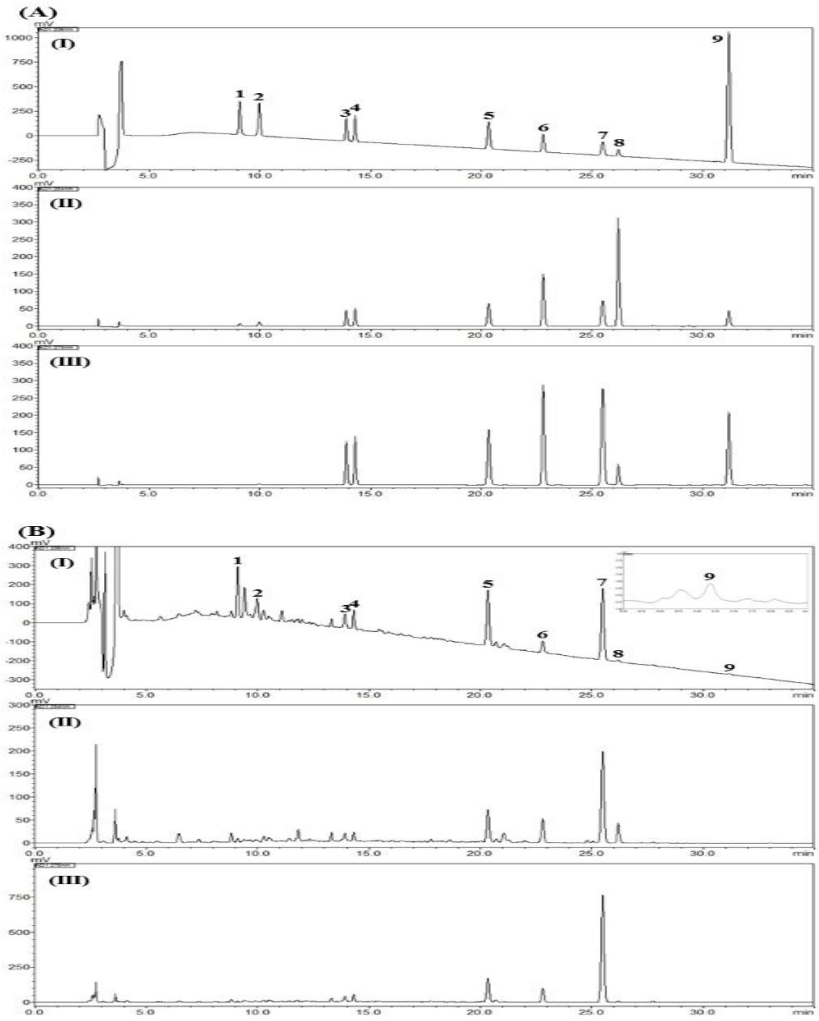

Figure 2: HPLC chromatogram of standard solution (A) and MT test solution at 206 nm (I), 254 nm (II), and 275 nm (III). Ephedrine HCl (**1**), amygdalin (**2**), liquiritin apioside (**3**), liquiritin (**4**), coumarin (**5**), cinnamic acid (**6**), cinnamaldehyde (**7**), glycyrrhizin (**8**), and 6-gingerol (**9**).

Cytotoxic effect of MT extract in RAW 264.7 cells

To evaluate the cytotoxicity of MT against RAW 264.7 cells, cells were exposed to various concentrations of MT water extract (15.625, 31.25, 62.5, 125, 250, 500, or 1000 μg/mL) for 24 h. As shown in Fig. 3, MT had no cytotoxic effect up to 500 μg/mL while reducing the viability by 34.43% at 1000 μg/mL of the treatment. Nontoxic concentrations of MT extract were used for subsequent *in vitro* assays.

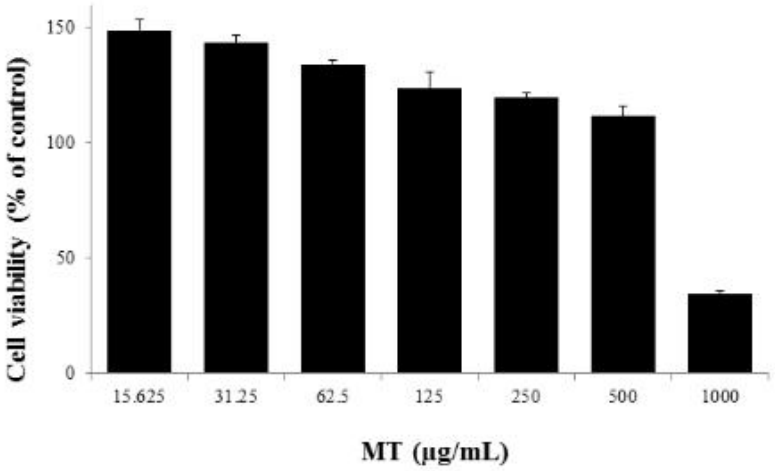

Figure 3: Cytotoxicity of MT in RAW 264.7 macrophages. Cells were exposed to various concentrations of MT extract (15.625, 31.25, 62.5, 125, 250, 500, or 1000 μg/mL) for 24 h. Cell viability (%) was assessed using a CCK-8 assay. The values are expressed as the mean ± S.E.M. of three independent experiments. MT: Mahwang-tang.

Inhibitory effects of the MT extract on the levels of inflammatory mediators in LPS-stimulated RAW 264.7 macrophages

We examined whether MT has anti-inflammatory effects by measuring production of NO and PGE_2. To induce *in vitro* inflammation, RAW 264.7 murine macrophages were stimulated with LPS (1 μg/mL). LPS stimulation significantly increased the level of NO (Fig. 4A). In contrast, MT treatment significantly reduced LPS-stimulated NO production at 250 and 500 μg/mL. In addition, LPS treatment significantly increased the amount of PGE_2 whereas MT dramatically suppressed LPS-mediated PGE_2 production in a dose-dependent manner (Fig. 4B). L-N^G-monomethyl arginine (NMMA) and indomethacin (IND) were used as positive controls for NO and PGE_2, respectively. In parallel, concurrent administration of LPS and MT extract suppressed the mRNA expression of iNOS and COX-2 in a dose-dependent manner compared with LPS alone (Fig. 5).

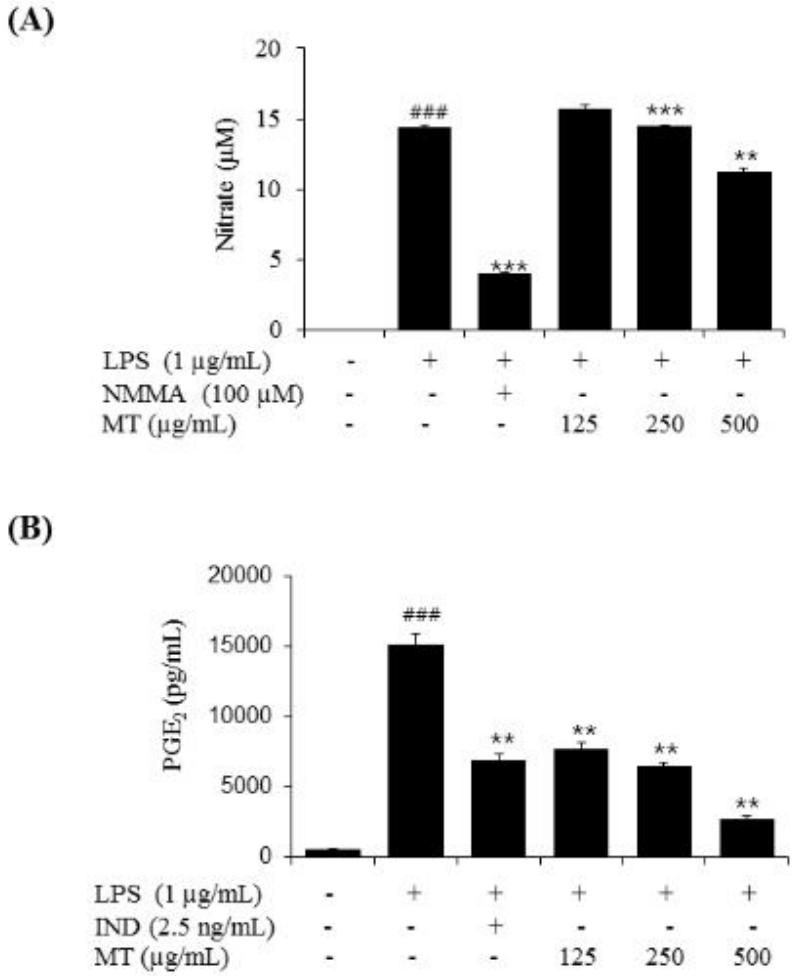

Figure 4: Effect of MT on LPS-stimulated NO and PGE_2 production in RAW 264.7 macrophages. The levels of NO (A) and PGE_2 (B) were measured in culture medium from cells pretreated with MT extract (125, 250, or 500 μg/mL) for 4 h and then stimulated with LPS (1 μg/mL) for an additional 20 h. NMMA (100 μM) and IND (2.5 ng/mL) were used as positive control drugs for (A) and (B), respectively. Each bar graph represents the mean of three independent experiments. $^{###}P < 0.001$ *vs.* vehicle control group; $^{***}P < 0.001$ or $^{**}P < 0.01$ *vs.* LPS-treated cells. MT: Mahwang-tang; NMMA: L-N^G-monomethyl arginine; and IND: indomethacin.

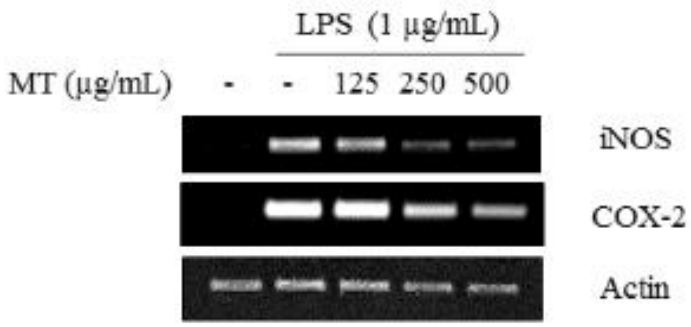

Figure 5: Effect of MT on the mRNA expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 macrophages. Cells were pretreated with MT extract (125, 250, or 500 μg/mL) for 1 h and then stimulated with LPS (1 μg/mL) for an additional 5 h. Total RNA was isolated from the cell pellets and subjected to RT-PCR for detecting iNOS and COX-2 mRNA expression. Levels of iNOS and COX-2 were adjusted by β-actin expression.

Conclusion

The established HPLC–DAD method for quantitative assessment of MT decoction was validated by the linearity, reproducibility, recovery, and precision validations. This method was anticipated to support the quality control of MT decoction or related herbal formulations. Furthermore, MT showed inhibitory effects on the production of inflammatory mediators NO and PGE_2 in LPS-activated RAW 264.7 macrophages. Consistently, MT suppressed LPS-stimulated expression of iNOS and COX-2 at the mRNA level. Our findings indicate the potent activity of MT as an anti-inflammatory drug candidate. Additional work will be necessary to confirm further its activity by analyzing the regulatory mechanisms using *in vitro* or *in vivo* models.

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Declaration of Conflict of Interest: Authors declare that there is no conflict of interest.

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