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Original Article

Comparison of the Ingredient Quantities, and Antioxidant and Anti-inflammatory Activities of *Hwangryunhaedok* Decoction Pharmacopuncture by Preparation Type

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[Abstract]

Objectives : The main aim of this study was to assess the comparative efficiency of two preparation types of *Hwangryunhaedok* decoction(HRHD-D) using distilled and mixed extraction by measuring the index components and indicators of antioxidant and anti-inflammatory effects.

Methods : The antioxidant activity was assessed by comparing distilled and mixed extractions of HRHD-D using an ELISA reader. The anti-inflammatory effect was determined by measuring NO amounts in RAW 264.7 cells. The contents were analyzed with high performance liquid chromatography-diode array detector(HPLC-DAD).

Results : The electron donating ability of mixed and distilled extractions obtained with 500 ppm DPPH(1,1-diphenyl-2-picrylhydrazyl assay) solution were 57.8 % and 4.2 %, respectively. The total phenolic content of mixed extraction was 6.9 times that of distilled extraction and total flavonoid content was 51.5 times higher. The anti-inflammatory effect was assessed by NO measurement, and was found to increase significantly dependent on concentration in all mixed extract concentrations(25, 50, 100, 200, 400 μg/mL), but the difference in distilled extraction by concentration was only significant at 200 and 400 μg/mL. The HPLC analysis results of mixed extract of HRHD-D showed detection of all four main active constituents of HRHD-D. However, they were not detected in the distilled extract of HRHD-D.

Conclusions : Mixed extraction with distillation added to decoction of HRHD-D showed better efficacy in antioxidant and anti-inflammatory effects, and ingredient quantities compared to distilled extraction. Further stability and clinical efficacy studies for standardization of mixed extractions are required.

Key words :
*H*wangryunhaedok decoctiom;
Pharmacopuncture;
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Ⅰ. Introduction

Herbal pharmacopuncture is a unique treatment approach that has diverged from the original form of acupuncture and moxibustion therapy of traditional oriental medicine. While pharmacopuncture is similar to traditional acupuncture in that it is used for both therapeutic and preventative purposes through physical stimulation of acupuncture points, pharmacopuncture is considered to be novel in its approach as it incorporates various aspects of traditional herbal medicine, meridianology, and acupuncture and moxibustion.

Hwangryunhaedok-decoction(HRHD-D) pharmacopuncture is a type of eight-principle pharmacopuncture, and the main ingredients are *Schisandra chinensis, Scutellariae radix*, *Phellodendron amurense* and *Copris japonica*, which are typically used for their anti-inflammatory, detoxification and pyretic effects. HRHD-D is also known as '*Orengedokuto*' in Japan and "*Huang-lian-jie-du-tang*" in China[1-3].

Pharmacopuncture clinically applies the theories of meridian and Qi/flavor. These theories are applied to many types of pharmacopuncture, including eight-principle, bee venom, meridian, and single-compound pharmacopuncture[4]. Of these, the ingredients for eight-principle pharmacopuncture are extracted using steam distillation based on the traditional properties and flavor(Qi/flavor theory) of the original decoction prescription ingredients as opposed to the pharmacological actions of specific bioactive ingredients within the herbal extracts.

Various types of pharmacopuncture are available for clinical use and they can be classified depending on the preparation process used in converting traditional herbal medicine to pharmacopuncture extracts. The main methods used in extracting active components from natural drug products and herbal ingredients for pharmacopuncture manufacture purposes are decoction, organic solvent extraction, and distillation after decoction.

The therapeutic effects of the distillation and decoction form of pharmacopuncture have already been demonstrated in several clinical studies. Pharmacopuncture treatment of distilled *Copris* japonica(黃連), one of the main herbs of HRHD-D pharmacopuncture, was reported by Park et al[5] to be effective for reducing pain in patients with cervical disc herniation. In addition, Yook et al[6] reported that distilled Rehmannia glutinosa, wild Ginseng and Astragali radix pharmacopuncture activated the autonomic nervous system, particularly the sympathetic nervous system, in healthy adult males. The effects of pharmacopuncture in distilled form have thus been indicated in a number of clinical trials.

Numerous clinical studies on the efficacy of the decoction form of HRHD-D have also been published. The reported effects include improving inflammation-mediated insulin resistance, inhibiting hypoxia-induced neuronal apoptosis and enhancing therapeutic potential in Alzheimer's disease[7-9]. These studies show that the distillation and decoction forms of pharmacopuncture are both efficacious.

The evaluation for standardization and safety assurance of the herbal extracts is achieved through analysis of the four active constituents mentioned above, and the results are used as basic data for standardization and safety assessment. While the efficacy and safety of HRHD-D pharmacopuncture in distilled form have been proved, quality control through ingredient analysis is still rendered difficult due to the distillation process. On the other hand, the biological activities and safety of the decoction form of HRHD-D have mainly been demonstrated in clinical trials, and quality control of ingredients achieved through rigorous lab experiments. For example, several methods have been reported for standard analyzation of the active constituents of traditional herbs, and these include *geniposide* of *Schisandra chinensis*, berberine of *Copris japonica*, *baicalin* from *Scutellariae radix*, and *palmatine* from *Phellodendron amurense* in HRHD-D[10,11].

We hypothesized that a mixed extraction of distillation and decoction would beget results reflecting higher effectiveness than when using only distilled extraction, and would thus be more widely applicable. In addition, the problem of ingredient

analysis for quality control in distilled extractions could be gotten round.

Accordingly, in this study, we compared the antioxidant and anti-inflammatory activities of the two types of HRHD-D preparations; (A) distilled extraction and (B) mixed extraction using distillation and decoction solvents, and suggest the possibility of improved HRHD-D pharmacopuncture quality control through analysis of ingredient quantities.

Ⅱ. Materials and methods

A. Preparation of HRHD-D samples

The four raw herbs included in HRHD-D, Schisandra chinensis, Copris japonica, Scutellariae radix and Phellodendron amurense used in this experiment were fully approved by the Korean ministry of food and drug safety(KFDA, Seoul, Korea). The composition ratio of the four different herbs, Schisandra chinensis, Copris japonica, Scutellariae radix and Phellodendron amurense, was set at 1:1:1:1, and 100 grams of the final product was obtained through the process.

The preparation types of extracts used were: (A) distilled extraction and (B) mixed extraction using both distillation and decoction. Preparation methods were as follows; (A) Distilled extraction: 25 grams of each raw herb was boiled in 1 liter of water at 105 °C for 3 hours. Thus, 100 mL of distilled extraction was obtained. The distilled extract was sterilized using an autoclave at 121 °C for 15 mins. (B) Mixed extraction using distillation and decoction: 25 grams of each raw herb was boiled in 1 liter of water. Distilled extraction(100 mL) was obtained through boiling and then sterilized for 15 mins at 121 °C. After distillation extraction, the entire volume was set at 3 liters by adding ethanol and decoction that was extracted at 105 °C for 3 hours. Excess water in the decoction extraction was evaporated and then the extracts were dissolved in 1 liter of 80 % ethanol. After precipitating in the refrigerator for 2 hours, ethanol was added to attain 2 liters of 90 % ethanol, and then stirred. After vaporization, the obtained powder extract was dissolved in 200 mL of water, and freeze-dried powder was used as samples. The two by-products, the sterile distilled extract samples and extract powder, were mixed together to be used as mixed extract samples.

B. Antioxidant activity

1. Chemicals and reagents

Folin-Ciocalteau reagent, gallic acid, quercetin, and 1,1-dipheny1-2-picrylhydrazyl(DPPH) were purchased from Sigma-Aldrich(USA). NaOH was purchased from Samchun(Korea). Na_2CO_3 and diethyleneglycol were purchased from Junsei(Japan).

2. DPPH radical scavenging test

The DPPH(1,1-diphenyl-2-picrylhydrazyl assay) radical-scavenging activity of pharmacopuncture was examined according to the method reported by Chang et al[12]. Test samples(100 μL) were mixed with 100 μL of 500 μg/mL DPPH-ethanol solution. After 30 mins of incubation at 37 °C, reduction of the DPPH (1,1-diphenyl-2-picrylhydrazyl assay) radical was measured by reading the absorbance at 540 nm using an ELISA reader. Three duplicates were made for each test sample. The inhibition ratio(per- cent) was calculated using the following equation:

% inhibition = [(absorbance of control − absorbance of sample) / absorbance of control] × 100

3. Determination of total polyphenol content

Total phenolic content of pharmacopuncture was determined using Folinc-Ciocalteau assay. To 100 μL of the test sample, 1 mL of 2 % Na_2CO_3 was added and allowed to sit for 2 mins. After 2 mins, 100 μL of 50 % Folin-Ciocalteau reagent was added. The mix- ture was incubated for 30 mins in the dark at 37 °C. After incubation, the absorbance was determined at 750 nm with an ELISA reader against a prepared reagent blank. The total phenolic content was calculated

based on the standard curve of gallic acid(10, 50, 100, 500, 1,000 μg/mL).

4. Determination of total flavonoid content

Diethyleneglycol(1 mL) was added and mixed using vortex to 100 μL of the test sample. After mixing, 1N NaOH 100 μL was added. The mixture was incubated in the dark for 30 mins at 37 °C. After incubation, the absorbance was determined at 415 nm with an ELISA reader against a prepared reagent blank. The total flavonoid was calculated on the basis of the standard curve of quercetin(10, 50, 100, 500, 1,000 μg/mL).

C. Anti-inflammatory activity

1. Chemicals and reagents

Lipopolysaccharide(LPS), thiazolyl blue tetrazolium bromide(MTT), sulfanilamide, and N-(1-naphthyl)-ethylenediamine dihydrochloride were purchased from Sigma-Aldrich(USA). Dulbecco's modified Eagle's medium(DMEM), fetal bovine serum(FBS) and antibiotic-antimycotic were purchased from Gibico BRL(USA). Dimethyl sulfoxide(DMSO) was purchased from Samchun(Korea). Sodium nitrite solution was purchased from Fluka(Switzerland). Phosphoric acid was purchased from Junsei(Japan).

2. Cell line and cell culture

RAW 264.7 murine macrophage cell line was purchased from the American type cell culture(KCLB, Seoul, Korea, No.40071). Cells were cultured in DMEM containing 10 % fetal bovine serum and 1 % antibiotic-antimycotic to subconfluent state. Cells were cultured at 37 °C in a humidified incubator with an atmosphere of 5 % CO_2.

3. Determination of nitric oxide(NO)

Nitrogen species activated in RAW 264.7 cells generate nitric oxide(NO), and the amount present in the cell culture medium in the form of NO^2- was determined by reaction with Griess reagent. RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^6 cells/mL, then in cubated at 37 °C in a 5 % CO2 incubator for 24 hrs. The cells were then simultaneously stimulated with LPS(1 μg/mL) and various concentrations(25, 50, 100, 200, 400 μg/mL) of test samples for 24 hrs. The supernatant was transferred, Griess reagent(0.1 %N-(1-naphthyl) ethylenediamide dihydrochloride, and 1 % sulfanilamide in 5 % phosphoricacid) added, and then the absorbance was measured with an ELISA reader at 540 nm. NO^2- concentration by a standard curve of sodium nitrite was used as index.

4. MTT assay for cell viability

RAW 264.7 cells were seeded at a density of 1×10^6 cells / mL in 96-well plates and incubated for 24 hrs, followed by treatment with the test samples(25, 50, 100, 200, 400 μg/mL) and LPS(1 μg/mL). MTT solution(1 mg/mL) was added to each well. After 4 hrs of incubation at 37 °C, the medium was discarded and the formazan blue formed in the cells was dissolved in DMSO. Optical density at 650 nm was determined with an ELISA reader(Sunrise, Tecan Austria GMBH). The percent of cells showing cytotoxicity was determined relative to the control group.

D. High performance liquid chromatography(HPLC) analysis

1. Reagents and standard materials

Water, acetonitrile and methanol were used as HPLC grades, which were purchased from JT Baker (USA). Phosphoric acid was purchased from Junsei (Japan). Indicator substances for geniposide(Schisandra chinensis, 梔子) and berberine(Copris japonica, 黃連) were purchased from WAKO pure Chemical Industries, Ltd(Japan), and those for baicalin(Scutellariae radix, 黃芩) and palmatine(Phellodendron amurense, 黃柏) were purchased from Sigma Aldrich Chemical Company, Inc(USA). The purity of these compounds was determined to be more than 95 % by HPLC analysis. All standard materials were prepared by dissolving in methanol.

2. Optimization of analytical conditions by HPLC-DAD

WatersTM 626 pump, 600s controller, Waters temperature control module, Waters In-line degasser, WatersTM 717plus autosampler and WatersTM 996 photodiode array detector(PDA) manufactured by Waters(USA) were used. The column used was TC-C18(2) column(5 μm, 4.6 ID × 250 mm, Aglient) and the column temperature was kept at 25 °C. The gradient elution of the mobile phase was applied as follows: mobile phase containing A(water containing 0.5 % phosphoric acid) and B(acetonitrile), linear gradient 22 % B for 0~10 min, 22-30 % B for 10~15 min, 30~40 % B for 15~30 min, 40~55 % B for 30~40 min, and 55~22 % B for 40~50 min. The flow rate was 1.0 mL/min. The injection volume was 30 μL. The four active constituents were detected by the 254 nm UV wavelength detector for simultaneous analysis.

a. Validation tests by HPLC

In this study, five differently concentrated aqueous solutions(× 1, × 1/2, × 1/4, × 1/8, × 1/16) were analyzed by HPLC method, and the analysis was carried out on each solution three times. Based on the outcome obtained through analysis, a calibration curve(y=ax + b) was constructed for the peak area(y) versus the concentration of standardized solution(x, μg/mL). Linearity was observed through the correlation coefficient(R^2) of the calibration curve, and the quantity of active constituents was assessed by setting the standard as linearity that has an R^2 value of over 0.99.

b. Application of HRHD-D samples by HPLC method

Index compounds for each herbal substance included in the distilled extracts and mixed extracts of HRHD-D, and actual contents were analyzed under established experimental conditions.

E. Statistical analysis

Each experiment was performed three times as separate experiments and the results are expressed as mean ± standard deviations(SD). The statistical significance of the results was evaluated using independent *t*-test, considering a p value of 〈0.05 as significant.

F. Ethics statement

N/A

Ⅲ. Results

A. Antioxidant activity

1. DPPH radical scavenging test

The electron donating ability(EDA) of the mixed extraction and distilled extraction was measured using 500 ppm DPPH solution, and the measurements were 57.8 and 4.2 %, respectively. These results demonstrate that the mixed extraction has a higher EDA than the distilled extraction(Table 1).

Table 1. Total Phenolic Content, Total Flavonoid Content and DPPH Radical Scavenging Activities by Pharmacopuncture Type

Type of pharmaco puncture	Total phenolic content (μg/mL)	Total flavonoid content (μg/mL)	DPPH radical scavenging activities(%)
Mixed extraction	177.13	502.25	57.7
Distilled extraction	25.80	9.75	4.2

2. Total phenolic content of the pharmacopuncture

In measuring total phenolic content, the regression equation was y=0.0015x+0.0367 between content of gallic acid(x) used as the standard reagent, and absorbance(y) at 760 nm. The increase in absorbance

dependent on increase of gallic acid content was significant(R^2=0.996). The total phenolic content of the mixed extraction and distilled extraction were 177.13 and 25.80 μg/mL, respectively. The total phenol content confirmed was about 6.9 times higher in the mixed extraction than the distilled extraction (Table 1).

3. Total flavonoid content of the pharmacopuncture

In measuring total flavonoid content, the regression equation was y=0.0004x+0.0011 between content of quercetin(x) used as the standard reagent and absorbance(y) at 415 nm. The absorbance increase dependent on increase of quercetin content was also significant(R^2=0.998). The total flavonoid content of the mixed extraction and distilled extraction were 502.25 and 9.75 μg/mL, respectively. Total flavonoid content was confirmed to be about 51.5 times higher in the mixed extraction than the distilled extraction (Table 1).

B. Anti-inflammatory activity

1. Effect of HRHD-D extracts on NO production

Nitric oxide(NO), a reactive nitrogen species, was recently recognized as an important factor acting in the inflammatory response. LPS-induced NO production in RAW 264.7 cells treated with concentrations of 25, 50, 100, 200, and 400 μg/mL of distilled and mixed extracts was assessed to compare the anti-inflammatory efficacy. As a result, the content of NO was 6 times higher in concentrations of Lipopolysaccharide(LPS)(1 μg/mL)-treated RAW 264.7 cells(control, CON) than the non-treated group (normal, NOR)(Fig 1a). In addition, decrease of NO in the mixed extracts was significantly concentration dependent. However, the difference in distilled extraction by concentration was significant only at 200 and 400 μg/mL(Fig 1a).

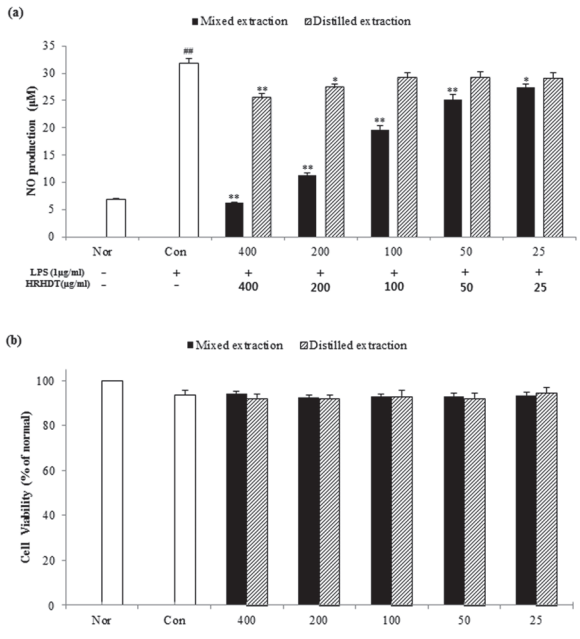

Fig. 1. Effects of HRHD-D on (a) RAW 264.7 macrophage-like cell viability and (b) NO production in RAW 264.7 macrophages stimulated with LPS; Bars represent the mean ± SD from experiments performed in triplicate

Solid columns(■) represent mixed extraction. Striped columns(▨) represent distilled extraction. Blank columns(□) represent normal and controlled conditions in mixed and distilled extraction. Normal(NOR) was treated with media only. Control(CON) was treated with LPS(1μg/mL) only.

: $p < 0.001$ indicates statistically significant differences from the normal group.

* : $p < 0.05$, ** : $p < 0.001$ indicates statistically significant differences when compared with the control group.

2. Effect of HRHD-D extracts on cell viability

The cytotoxicity was measured to determine whether inhibition of NO production was caused by cytotoxicity using the thiazolyl blue tetrazolium bromide (MTT) assay. As a result, RAW 264.7 cells treated with HRHD-D extracts were confirmed to show non-toxicity as the survival rate was not affected in LPS(1 μg/mL)-treated cells at all con- centrations (Fig 1b).

C. Analytical results by HPLC-DAD

1. Validation data

In this study, HPLC method was validated according to the European Medicines Agency(EMA) guidelines[13]. The calibration curve for each standard substance was generated by comparing the peak area(y) versus the concentration rate of the standard solution(x, ㎍/mL). The peak area of the calibration curve for each active constituent of geniposide, baicalin, palmatine and berberine was observed and presented without interference in special nested experiments. Thus, simultaneous analysis of the four main active constituents was possible. Geniposide, baicalin, palmatine and berberine were detected at 5.2, 22.6, 24.5 and 25.1 mins, respectively. All of these materials were detected within 30 minutes(Fig. 2). The linearity

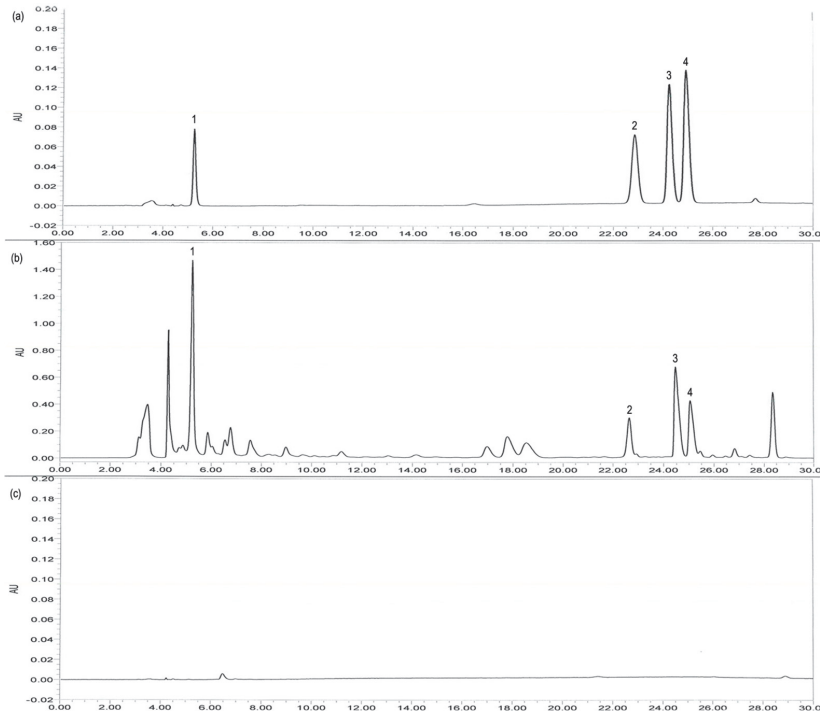

Fig. 2. HPLC chromatogram by preparation method
(a) standard mixture.
(b) mixed extraction of HRHD-D.
(c) distilled extraction of HRHD-D.
1 : geniposide. 2 : baicalin. 3 : palmatine. 4 : berberine.

Table 2. The Linearity, Regression Equation, Correlation Coefficient(R^2), and LOD and LOQ for Measurement of the Four Standard Components by HPLC

Components	Regression equation	R^2 (n=3)	LOD (㎍/mL)	LOQ (㎍/mL)
Geniposide	Y=14375x + 30557	0.9992	0.33	1.01
Baicalin	Y=26286x + 76369	0.9999	1.29	3.92
Palmatine	Y=72829x + 329243	1.0000	0.07	0.20
Berberine	Y=68157x - 144551	0.9993	0.43	1.29

Y : peak area. x : amount(㎍/mL).
LOD : limit of detection = 3.3 × (SD of the response / slope of the calibration curve).
LOQ : limit of quantitation = 10× (SD of the response / slope of the calibration curve).

of calibration curve showed good correlation coefficient(R^2) values of 0.999 or higher with a wide range(0.17~1.68 μg/mL). The values of limit of detection(LOD) and limit of quantitation(LOQ) were 0.07 ~ 1.29 μg/mL and 0.20 ~ 3.92 μg/mL, respectively(Table 2). The values of relative standard deviation(%) for accuracy and precision of within-run and between-run analysis ranged from 0.19 % to 0.57 % and from 0.10 % to 2.09 %, respectively. It was then standardized to standard compound solution and results were measured repeatedly 4 times a day(n=4 times/day). Between-run was measured three times on days 1, 3, 7(n=3 days, 4 times/day). These results show that the developed method was sufficiently

Table 3. Within-run and Between-run Variations of 4our Standard Materials by HPLC-DAD

Standard materials	Retention time (min)	Concentration (μg/mL)	Within-run (n=4)		Between-run (n=3)	
			Mean ± SD (μg/mL)	RSD(%)[a]	Mean ± SD (μg/mL)	RSD(%)[a]
Geniposide	5.2	25	24.71 ± 0.43	0.43	24.32 ± 1.53	2.09
		50	50.43 ± 1.08	0.53	50.14 ± 2.18	1.45
		100	99.86 ± 1.50	0.37	99.96 ± 2.96	0.99
Baicalin	22.6	125	126.10 ± 2.27	0.45	126.00 ± 1.40	0.37
		250	248.35 ± 1.80	0.18	247.31 ± 0.27	0.10
		500	500.55 ± 6.65	0.33	500.50 ± 2.74	0.18
Palmatine	24.5	75	76.00 ± 0.50	0.16	75.21 ± 3.23	1.43
		150	148.50 ± 1.07	0.18	147.25 ± 5.5	1.26
		300	300.50 ± 0.20	0.02	299.62 ± 10.46	1.16
Berberine	25.1	12.5	12.69 ± 0.19	0.37	12.94 ± 0.04	0.10
		25	24.71 ± 0.43	0.43	24.35 ± 0.11	0.15
		50	50.10 ± 0.54	0.27	50.22 ± 1.87	1.24

RSD : relative standard deviation.
a : RSD (%) = (SD / Mean) × 100.

Table 4. Analytical Results of Accuracy Test for Four Standard Materials by HPLC

Components	Spiked amount (μg/mL)	Measured amount (μg/mL, n=4)	RSD (%)	Recovery[a] (%)
Geniposide	25	24.92	0.41	99.66
	50	50.13	0.36	100.25
	100	99.96	0.40	99.96
Baicalin	125	126.10	0.52	100.88
	250	248.35	0.19	99.34
	500	500.55	0.34	100.11
Palmatine	75	75.95	0.57	101.26
	150	148.58	0.27	99.05
	300	300.47	0.22	100.16
Berberine	12.5	12.69	0.44	101.54
	25	24.71	0.47	98.85
	50	50.10	0.28	100.19

a : Recovery (%)=[(amount found-original amount)/amount spiked] × 100 %

accurate and yielded reproducible results in HRHD-D samples in this study(Table 3, 4).

2. Application of HRHD-D samples

The distilled extraction(A) and mixed extraction(B) of HRHD-D were analyzed through HPLC methods to determine the main active constituents of HRHD-D (Fig. 2). The four active constituents were detected in the mixed extraction of HRHD-D, but not in the distilled extraction of HRHD-D. Therefore, determination and analysis of all four main active constituents of HRHD-D using the HPLC method was possible only in the mixed extraction.

Ⅳ. Discussion

In this study, experiments were carried out to assess ingredient quantities and antioxidant and anti-inflammatory activities in order to compare the efficacy of mixed extractions and distilled extractions of HRHD-D.

In this study, we measured EDA using DPPH solution. As a result, mixed extractions were revealed to have higher EDA than distilled extractions. Additionally, the mixed extraction had a total phenolic and flavonoid content 6.9 and 51.5 times higher, respectively, than that of the distilled extraction. Baicalin, the main ingredient of one of the herbs of HRHD-D, Scutellaria baicalensis, is a type of flavonoid and known as an excellent antioxidant, and has been demonstrated to inhibit oxidation through several studies. It was published that its actions include elimination of such radicals as DPPH and ABTS(3-ethyl-benzothiazoline-6-sulfonic acid)[14,15]. It has also been demonstrated that geniposide of Schisandra chinensis, one of the major iridoids glycosides, has antioxidant and anti-inflammatory effects[16,17]. As a result, it can be deduced that mixed extraction adding decoction of HRHD-D to distillation has a higher efficiency than only distillation.

In this study, we compared the anti-inflammatory effects of mixed extractions and distilled extractions through inhibition experiments by measuring inflammatory mediators in LPS(1 μg/mL)-treated RAW 264.7 cells(Control, CON). As a result, mixed extraction with decoction and distillation of HRHD-D was shown to demonstrate higher anti-inflammatory activity then distillation only. A major ingredient of Copris japonica, berberine, and a main ingredient of Phellodendron amurense, palmatine, are alkaloids whose anti-inflammatory properties of cortex phellodendri amurensis in vitro and in vivo may explain its utility for attenuating inflammation-related diseases and effectively inhibiting bacteria, fungi and viruses[18,19]. Additionally, it was reported by Peng et al[20] that Gardenia jasminoides(Schisandra chinensis) showed strong inhibitory effects on NO production when evaluated for its effect using macrophages.

In this study, the comparison between mixed extraction and distilled extraction was made by evaluating the main active constituents of HRHD-D. The main herbal ingredients of HRHD-D are Schisandra chinensis, Copris japonica, Scutellariae radix and Phellodendron amurense. The main active ingredients of HRHD-D are genioposide, palmatine, berberine and baicalin and they are detected in the same condition of the mobile phase. They are detected simultaneously at a UV wavelength of 254 nm within 30 minutes(Fig. 2a). The calibration curve showed good correlation coefficient(R^2) with values of 0.999 or higher. The values of LOD and LOQ were 0.07~1.29 μg/mL and 0.20~3.92 μg/mL, respectively(Table 2).

In the sample experiments, mixed extraction and distilled extraction of HRHD-D pharmacopuncture were analyzed to detect individual active constituents of HRHD-D through HPLC method. The four main active constituents of HRHD-D were all detected in the mixed extract of HRHD-D(Fig. 2b). However, they were not detected in the distilled extract of HRHD-D(Fig. 2c).

Ⅴ. Conclusion

We observed the differences in efficacy and analysis results in ingredient quantities, and anti-oxidant and anti-inflammatory effects of HRHD-D pharmacopuncture according to preparation method. Mixed extraction with distillations added to decoction of HRHD-D showed better efficacy in antioxidant and anti-inflammatory effects in comparison with distillation only extraction, and the contents of each herb could be detected though indicator ingredient quantities. Therefore, application of mixed extractions could be seen as highly beneficial as it can enhance the efficacy of HRHD-D pharmacopuncture treatment, as well as enable standardization through detection of indicator ingredients. However, some major disadvantages of mixed extraction are that the preparation process is quite complex and requires a lot of time and attention. Several clinical studies on the efficacy of distillation and decoction types of HRHD-D pharmacopuncture have been conducted[21,22]. However, no clinical research has been performed on the effect of mixed extractions of HRHD-D with distillation added to decoction, and studies on safety are still insufficient. Consequently, further stability and clinical efficacy studies for standardization measures of mixed extraction are required.

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