

Determination of Five Chemical Markers in DF Formula, the Herbal Composition of *Ephedra intermedia*, *Rheum palmatum*, and *Lithospermum erythrorhizon*, Using High-performance Liquid Chromatography-ultraviolet Detection

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ABSTRACT

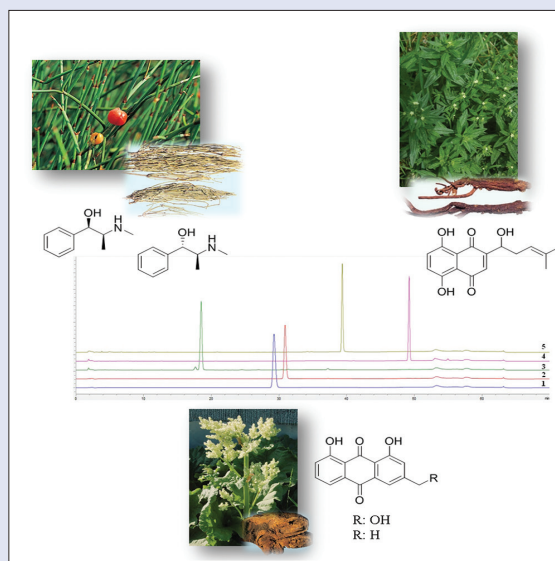
Background: DF formula is a herbal preparation comprised three medicinal herbs, namely, *Ephedra intermedia*, *Rheum palmatum*, and *Lithospermum erythrorhizon*, which is being used for the treatment of obesity and liver fibrosis in Korean local clinics. **Objective:** Since the abovementioned three herbs exist with different proportions in DF formula and their chemical markers have different physicochemical properties; it is quite challenging to develop an analytical methodology for the determination of these chemical markers. **Materials and Methods:** For the analysis of the three herbs, five chemicals, (+)-pseudoephedrine (1) and (-)-ephedrine (2) for *E. intermedia*, aloe-emodin (3), and chrysophanol (4) for *R. palmatum*, and shikonin (5) for *L. erythrorhizon*, were selected for method validation of DF formula, and the analytical conditions were optimized and validated using high-performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV). **Results:** The specificities for the five compounds 1–5 were determined by their UV absorption spectra (1–4: 215 nm and 5: 520 nm). Their calibration curves showed good linear regressions with high correlation coefficient values ($R^2 > 0.9997$). The limits of detection of these five markers were in the range 0.4–2.1 ng/mL, with the exception of 5 (12.7 ng/mL). The intraday variability for all the chemical markers was less than a Relative standard deviation (RSD) of 3%, except for 5 (RSD = 12.6%). In the case of interday analysis, 1 (1.0%), 2 (3.1%), and 4 (3.7%) showed much lower variabilities (RSD < 5%) than 3 (76%) and 5 (8.2%). Moreover, the five chemical markers showed good recoveries with good accuracies in the range of 90%–110%. **Conclusions:** The developed HPLC-UV method for the determination of the five chemical markers of the components of DF formula was validated.

Key words: *Ephedra intermedia*, high-performance liquid chromatography coupled with an ultraviolet detector, *Lithospermum erythrorhizon*, method validation, *Rheum palmatum*

SUMMARY

- DF formula, the herbal composition of *Ephedra intermedia*, *Rheum palmatum* and *Lithospermum erythrorhizon*
- Five chemical markers in DF formula were (+)-pseudoephedrine (1) and (-)-ephedrine (2) for *E. intermedia*, aloe-emodin (3) and chrysophanol (4) for *R. palmatum*, and shikonin (5) for *L. erythrorhizon*, with quite different physicochemical properties

- Five chemical markers in DF formula were determined by HPLC-UV



Abbreviations used: EP: (-)-ephedrine; PSEP: (+)-pseudoephedrine; HPLC: High-performance liquid chromatography; UV: Ultraviolet; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation.

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INTRODUCTION

DF formula (“Gang-Ji-Hwan” in Korean), a herbal preparation comprised three medicinal herbs, *Ephedra intermedia* Schrenk, *Rheum palmatum* Linne, and *Lithospermum erythrorhizon* Siebold et Zuccarini, is currently being used for the treatment of obesity and liver fibrosis in Korean local clinics. The sources for these three herbs in the DF formula are recorded in the 11th Korean Pharmacopoeia. *E. intermedia* (EI, Ephedraceae) is a medicinal herb native to Northeastern China, Russia, and

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Mongolia and has been used for the treatment of asthma and cough for thousands of years in Eastern Asia.^[1] The six major active components of the *Ephedra* species are (-)-ephedrine (EP), (+)-pseudoephedrine (PSEP), (-)-*N*-methylephedrine, (+)-*N*-methylpseudoephedrine, (-)-norephedrine, and (+)-norpseudoephedrine.^[2] These alkaloids act on the sympathomimetic agonists at adrenergic receptors and stimulate the central nerve system, causing an increase in cardiac rate and contractility, bronchodilation, and peripheral vasoconstriction.^[3] *R. palmatum* (RP, Polygonaceae) is a perennial herb used for purgation, purging heat, promoting blood circulation, and removing blood stasis from ancient China.^[4,5] The major bioactive compounds of RP are anthraquinone derivatives such as emodin, aloe-emodin, chrysophanol, and physcion, which are naturally occurring laxatives.^[6] *L. erythrorhizon* (LE, Boraginaceae), a perennial plant native to Eastern Asia, has been used as a natural colorant for food and clothes as well as a medicinal herb for the treatment of skin-related afflictions such as burns, acne, pigmentation changes, and atopic dermatitis.^[7,8] Naphthoquinone derivatives shikonin, acetylshikonin, and isobutylshikonin are the bioactive compounds obtained from LE.^[9] In particular, shikonin increases the glucose uptake in skeletal muscle cells and improves plasma glucose levels in diabetes-induced rats.^[10] In this work, a validation study of the five chemicals in DF formula has been performed by high-performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV) analysis. DF formula is a herbal extract prepared with different proportions of the three above-mentioned herbs. As DF formula comprised three different types of chemical markers, namely, ephedra alkaloids for EI, anthraquinones for RP, and naphthoquinones for LE, it is challenging to optimize the HPLC analytical conditions for their simultaneous determination.

Experimental section

Material and reagents

DF formula, *E. intermedia* (EI, 1 kg), *R. palmatum* (RP, 1 kg) and *L. erythrorhizon* (LE, 1 kg) were received as a gift from Dr. Soon Shik Shin, at the Department of Korean Medicine, Dong-eui University, who also identified all of these samples. The three herbs were deposited in the Herbarium of the College of Pharmacy, Kangwon National University (KNUPH-EI-1, KNUPH-RP-1, and KNUPH-LE-1). HPLC grade solvents were purchased from TEDIA (Fairfield, OH, USA). Ethanol, formic acid, sodium dodecyl sulfate (SDS), EP, PSEP, and shikonin were purchased from Sigma-Aldrich (St. Louis, MO, USA) while aloe-emodin and chrysophanol were obtained from Corescience Inc. (Seoul, Korea). The purities of the standards were >98.0%.

Sample preparation

The DF formula was prepared by the patented technologies. Briefly, the three herbs EI, RP, and LE were chopped into small pieces of size 2–3 cm and mixed in the ratio of 80:9:11. Subsequently, 2 g of the mixed material was dissolved in 10 mL of 70% ethanol and extracted using the Soxhlet technique for 4 h at 65°C. The mixture was then filtered and evaporated to dryness using a rotary evaporator. After freeze-drying to a powder, it was dissolved in 1 mL of 70% methanol for the analyses of compounds 1–4, in EI and RP. For the analysis of 5, 100 mg of the freeze-dried powder was suspended in water, followed by fractionation with *n*-hexane (2 × 10 mL). After the *n*-hexane fraction was evaporated and freeze-dried, 30 mg of the residue was dissolved in 1 mL of acetonitrile. All the samples were filtered through a 0.45-μm polyvinylidene fluoride membrane filter before injection into an HPLC.

High-performance liquid chromatography coupled with an ultraviolet detector analysis

The HPLC analyses were performed with an Agilent 1260 infinity HPLC-UV system consisting of a binary pump, an autosampler,

a thermostated column compartment, and a UV detector (Agilent Technologies Mfg GmbH and Co. KG, Waldbronn, Germany). For chromatographic analysis, a HECTOR-M C18 column (250 mm × 4.6 mm i. d.; 5 μm, RStech, Daejeon, Korea) with a compatible Phenomenex guard column (4 mm × 3 mm i. d.; 5 μm) was used for the quantification and qualification of chemical markers in the samples. Wavelengths for 1–4 and 5 were at 215 and 520 nm, respectively. The mobile phase for compounds 1–4 in DF formula comprised 25 mM SDS in water (A) and acetonitrile (B) using the following gradient system: isocratic 60% A at 0–25 min, linear gradient 60%–40% at 25–35 min, isocratic 40% at 35–40 min, linear gradient 40%–20% at 40–50 min, and isocratic 20% at 50–60 min. Compound 5 in the standard solution and DF formula was analyzed using an isocratic mixture of 50% of 0.1% formic acid in water and 50% acetonitrile as the mobile phase for 30 min. The flow rate was 1.0 mL/min, and aliquots of 10 μL were injected using the autosampler for the analyses.

Method validation

For linearity and sensitivity studies, the five compounds were diluted to the appropriate concentrations based on preliminary studies. A range of five concentrations of each standard was determined under the optimized analytical conditions by performing three identical experiments. The limits of detection (LOD) and quantification were determined under the chromatographic analysis at signal-to-noise ratios of 3 and 10, respectively. For the precision study, the intraday variabilities of the five compounds were tested by preparing their solutions in three different concentrations and examining them within 1 day and the interday variabilities were analyzed for a single concentration over 3 days (first, third, and fifth day), respectively. Meanwhile, the accuracies were determined by the percentages of the recovered amount of each compound in DF formula after the addition of standards (100, 200, and 300 ppm). The relative standard deviation (RSD) was taken to determine of precision of accuracy.

RESULTS AND DISCUSSION

Optimization of high-performance liquid chromatography coupled with a ultraviolet detector conditions for the chemical markers

DF formula has been used to treat ailments such as obesity and liver fibrosis in Korean local clinics, but it has not been validated to date. In the present study, the optimization of analytical conditions and development of method validation have been attempted for the determination of the chemical markers for DF formula. It is noteworthy that DF formula is an herbal preparation containing different proportions of three different herbs (EI, RP, and LE), which have chemical markers with different physiochemical properties.

Since EI accounted for the largest proportion of the DF formula, PSEP (1) and EP (2), the two ephedra alkaloids which are the major components in EI were studied first.^[11] The solvent system buffered with SDS was found to be suitable for reproducibility in routine analysis, without the deterioration of peaks for 1 and 2.^[12] Negative ions in SDS neutralized the positive ions in ephedra alkaloids, which showed good retention on the HPLC column and separated from the other components with high resolution. After testing various concentrations of SDS with the MeOH extract of EI (10 mg/mL), it was found that the optimal solvent system was acetonitrile-water with 25 mM SDS. Subsequently, the solvent gradient system was adjusted and optimized for the chemical markers of RP and LE. Using the optimized solvent gradient conditions, aloe-emodin (3) and chrysophanol (4), which are anthraquinone derivatives without sugar moieties, and shikonin (5), which is a simple naphthoquinone derivative with dimethylallyl moiety were found to be

well-separated from other peaks [Figures 1 and 2]. The five chemicals, namely, two ephedra alkaloids (1 and 2) for EI, two anthraquinones (3 and 4) for RP, and a naphthoquinone (5) for LE, were selected for the method validation of DF formula.

Optimization of high-performance liquid chromatography coupled with an ultraviolet detector conditions for DF formula

The optimized conditions were utilized to verify the existence of the above-mentioned five markers in DF formula. As shown in Figure 3, four compounds, 1–4 could be detected except 5 from LE. Compound 5 is a naphthoquinone which is highly susceptible to external stimuli such as light, temperature, oxygen, and pH.^[13,14] It was found that 5 was either not detected or its concentration was under the limit of detection owing to its possible degeneration at the high extraction temperature (65°C) and the low amount ratio of LE in the DF formula. Thus, the DF formula was

suspended in distilled water and fractionated with *n*-hexane to alleviate the extraction yield of compounds with lower polarity. Since 5 was not detected in the solvent system optimized for EI and RP in DF formula, 5 could be detected in the optimized analytical condition using another optimized mobile phase consisting of 0.1% formic acid and acetonitrile and at 520 nm in HPLC-UV [Figure 4].

Method validation

Specificities for the four compounds were determined by their UV absorption spectra [Figure 5], which indicated the singularity of each peak. The maxima for EP and PSEP were verified at 215 nm, while those for AE and CP were at 215 and 254 nm, respectively, and for SH at 215 and 520 nm. Thus, the chemical markers for EI and RP were measured at 215 nm and for LE at 520 nm under each analytical condition.

Five calibration curves were calculated using the five chemical markers in a wide range of concentrations (20–1000 µg/mL for 1–3, 10–500 µg/mL for

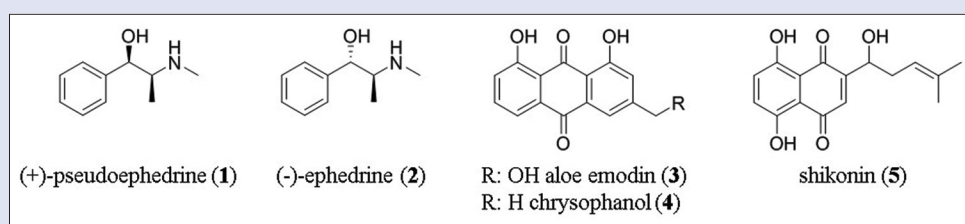


Figure 1: Chemical structures of the five chemical markers

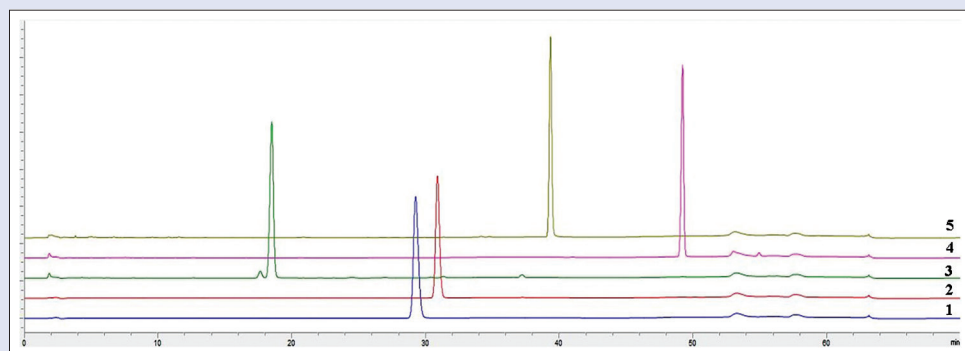


Figure 2: High-performance liquid chromatography chromatograms of chemical markers 1-5. 1: (+)-pseudoephedrine; 2: (-)-ephedrine; 3: aloes emodin; 4: chrysophanol; 5: shikonin

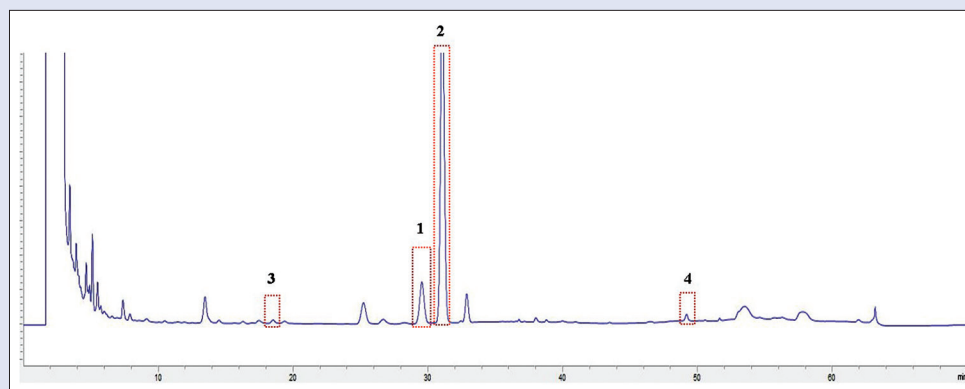


Figure 3: High-performance liquid chromatography chromatograms of DF formula. 1: (+)-pseudoephedrine; 2: (-)-ephedrine; 3: aloes emodin; 4: chrysophanol

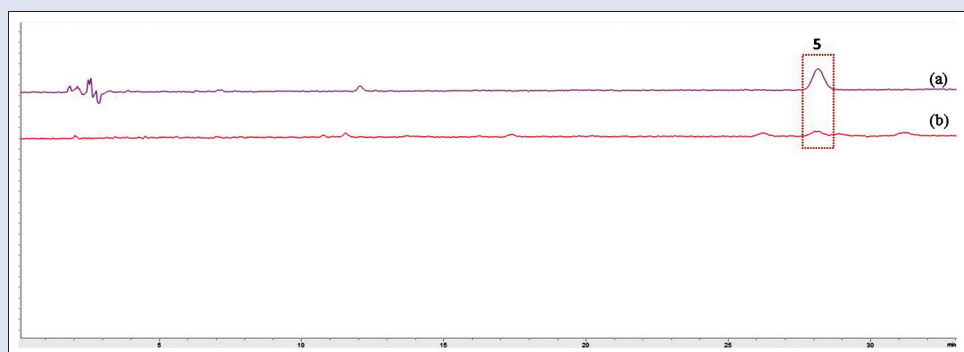


Figure 4: High-performance liquid chromatography chromatograms of (a) 5 and (b) DF formula (5: shikonin)

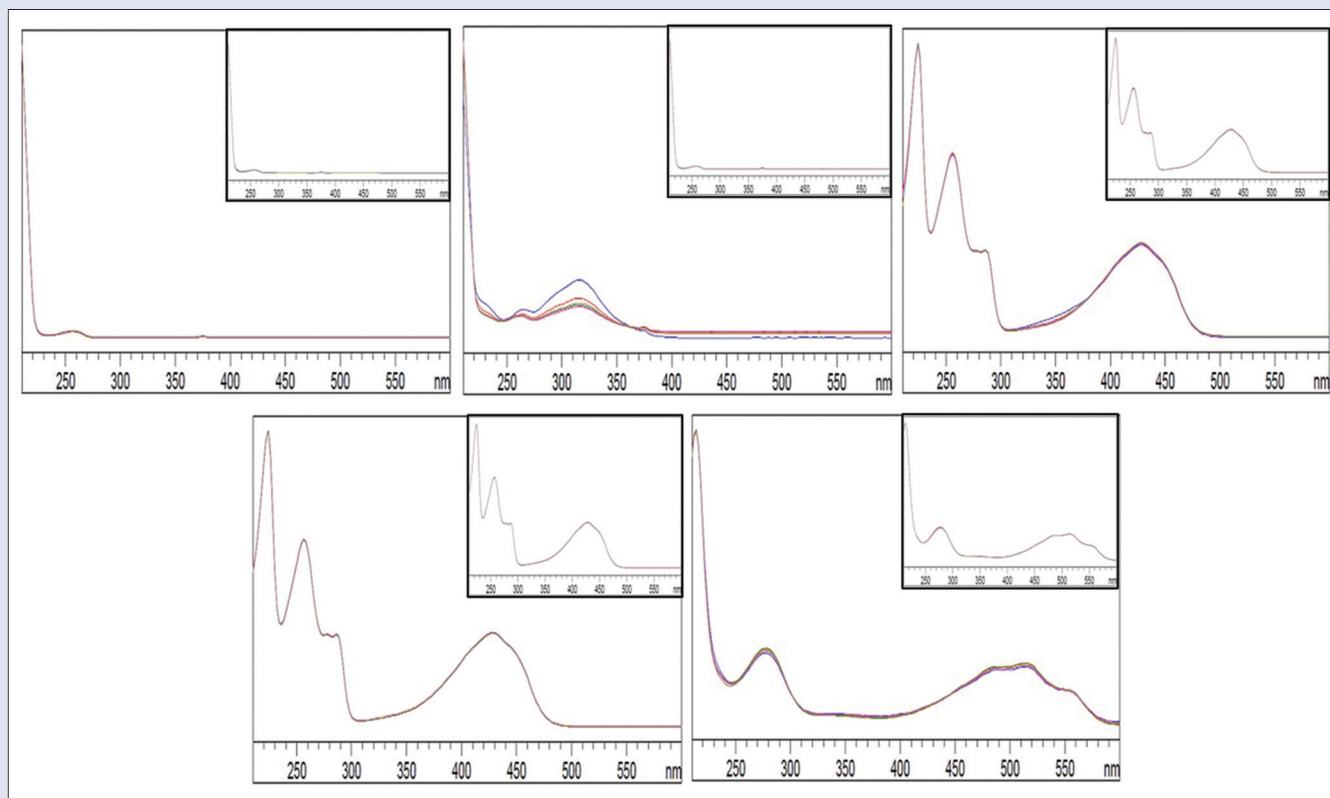


Figure 5: Ultraviolet spectra of compounds 1–5 (a–e), respectively, acquired with a diode array detector in standards (smaller boxes) and DF formula

4, and 5–250 µg/mL for 5) and all of these showed good linear regressions with high correlation coefficient values ($R^2 > 0.9997$) [Table 1]. The LODs of the five markers were in the range 0.4–2.1 ng/mL with the exception of 5 (12.7 ng/mL), and these displayed high sensitivities under the optimized chromatographic condition. The higher LOD of 5 compared to compounds 1–4, may be a result of its structural deterioration under external stimuli such as light and temperature.

A precision test was also conducted by evaluating the intra- and inter-day variances for the five markers. The intraday test was assayed at three concentrations on the same day and those for the interday on three different days (first, third, and fifth day) [Table 2]. The intraday variability for all the chemical markers was less than the RSD of 3% with the exception of SH (12.6%). In the case of interday variance, compounds 1 (1.0%), 2 (3.1%), and 4 (3.7%) showed much lower variabilities (RSD < 5%) than 3 (7.6%) and 5 (8.2%). Nevertheless, the

five chemical markers showed good recoveries with good accuracies (in the range of 90%–110%) and were found to be stable.

CONCLUSIONS

In this study, the determination method of five chemical markers in DF formula was successfully established. The analytical conditions were optimized according to the physicochemical properties of the chemical markers and different proportions of three herbs in DF formula. Four chemical markers (1–4) were simultaneously determined using the solvent system buffered with 25 mM SDS for 1 and 2, which possess a secondary amine group and are positively charged. Compound 5 could not be detected in the optimized analytical condition owing to its small quantity in DF formula and its instability toward external stimuli. Therefore, a selective extraction method for 5 was developed and optimized. This method enabled the detection and validation of 5. These

Table 1: Linear regression data, limits of detection, and limit of quantification of the five chemical markers 1-5 present in DF formula

| Compounds | Retention time | Linear regression equation | R ² | LOD (µg) | LOQ (µg) |
|----------------|----------------|----------------------------|----------------|----------|----------|
| 1 ^a | 28.4 | y=16,094.8252x-1.0821 | 1.0000 | 0.0015 | 0.0044 |
| 2 ^a | 30.8 | y=12,072.2485x+65.3639 | 0.9997 | 0.0021 | 0.0063 |
| 3 ^a | 18.5 | y=14,399x+0.0732 | 1.0000 | 0.0005 | 0.0016 |
| 4 ^a | 49.2 | y=12,522x-25.531 | 0.9999 | 0.0004 | 0.0012 |
| 5 ^b | 24.8 | y=52,266x+11.055 | 1.0000 | 0.0127 | 0.0385 |

^aCompounds 1-4 were analyzed under the mobile phase consisted of 25 mM SDS in water and acetonitrile using the following gradient system: Isocratic 60% A at 0-25 min, linear gradient 60%-40% at 25-35 min, isocratic 40% at 35-40 min, linear gradient 40%-20% at 40-50 min, and isocratic 20% at 50-60 min;

^bCompound 5 was analyzed by the isocratic mixture of 50% of 0.1% formic acid in water and 50% acetonitrile for 33 min. SDS: Sodium dodecyl sulfate; LOD: Limits of detection; LOQ: Limit of quantification

Table 2: Precision and accuracy data of compounds 1-5 in DF formula

| Compounds | Precision | | | | Accuracy | | |
|-----------|-------------|---------|-------------|---------|--------------------|--------------|---------|
| | Intraday | | Interday | | Spiked amount (µg) | Accuracy (%) | RSD (%) |
| | Amount (µg) | RSD (%) | Amount (µg) | RSD (%) | | | |
| 2 | 8.10 | 0.78 | 5.81 | 1.03 | 7.90 | 102.48 | 0.78 |
| | 5.51 | 1.21 | | | 5.40 | 101.95 | 1.21 |
| | 3.89 | 0.28 | | | 3.90 | 99.8 | 0.28 |
| 3 | 0.58 | 1.11 | 0.14 | 7.61 | 0.57 | 102.12 | 1.11 |
| | 0.32 | 1.67 | | | 0.32 | 100.85 | 1.67 |
| | 0.19 | 1.91 | | | 0.19 | 100.25 | 1.91 |
| 3 | 1.39 | 0.88 | 0.72 | 3.06 | 1.36 | 101.97 | 0.88 |
| | 0.90 | 2.10 | | | 0.86 | 104.81 | 2.10 |
| | 0.62 | 2.37 | | | 0.61 | 101.91 | 2.37 |
| 4 | 0.74 | 0.54 | 0.54 | 3.75 | 0.77 | 95.68 | 0.54 |
| | 0.50 | 0.26 | | | 0.52 | 96.77 | 0.26 |
| | 0.36 | 0.30 | | | 0.37 | 97.52 | 0.30 |
| 5 | 0.11 | 7.32 | 0.04 | 8.22 | 2.68 | 91.63 | 7.32 |
| | 0.07 | 7.81 | | | 1.17 | 93.78 | 7.81 |
| | 0.04 | 10.21 | | | 0.70 | 97.66 | 10.21 |

results can also be used for the method validation of other components of DF formula and provide scientific information for further exploration of herbal medicines that comprised several herbs or have unstable naphthoquinone derivatives.

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Conflicts of interest

There are no conflicts of interest.

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