and the cross-peak of δ_H 7.61 with δ_C 114.5 and 151.2 revealed the oxygenated substituents on C-3 and C-4 of benzoic acid. Additionally, the cross-peak of methyl proton δ_H 3.89 and anomeric proton δ_H 5.00 (1H, J=7.5 Hz) with δ_C 150.1 and δ_C 151.2, respectively revealed the methoxy group at C-3 and the *O*-glucosyl at C-4. Thus, compound **6** was identified as vanillic acid 4-*O*-*β*-D-glucopyranoside [11].

4. Conclusion

A phytochemical investigation of *C. orchioides* collected in Vietnam led to the isolation of six phenolic compounds. They were identified as curculigoside A (**1**), curculigoside B (**2**), orcinol *O*-*β*-D-glucopyranoside (**3**), orcinol *O*-gentiobioside (**4**), glucosyringic acid (**5**) and vanillic acid 4-*O*-*β*-D-glucopyranoside (**6**). Compound **6** was found in *C. orchioides* for the first time.

Acknowledgment: *This research is partly funded by Daknong Department of Science and Technology.*

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Journal of Medicinal Materials, 2023, Vol. 28, No. 2 (pp. 93 - 98)

QUANTITATIVE DETERMINATION OF DAPHNORETIN IN AERIAL PARTS OF *WIKSTROEMIA INDICA* L. (THYMELAEACEAE) BY HPLC-DAD

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(Received March 26th, 2023)

Summary

Quantitative Determination of Daphnoretin in Aerial Parts of *Wikstroemia indica* L. (Thymelaeaceae) by HPLC-DAD

A simple and sensitive HPLC method was proposed for the quantification of daphnoretin in aerial parts of *Wikstroemia indica* (L.) (*W. indica*). The chromatographic separations were obtained by an Agilent C_{18} column (250 x 4.6 mm, 5 µm particle size) using isocratic elution with acetonitrile and water (40/60, v/v), at a flow rate of 0.6 mL/min, with an injection volume of 10 µL and detection wavelength of 345 nm. The calibration curve displayed a good linear relationship (linearity ranging from 14.88 - 238 µg/mL and R^2=0.9999). The method was validated for system suitability (RSD of peak area = 0.21%; RSD of t_R = 0.15%); precision (RSD < 3.0%) and accuracy (recovery: 95.60-102.92%). The validation results displayed good accuracy, repeatability, linearity, and selectivity according to the ICH guideline. The percentage content of daphnoretin in aerial part samples of *W. indica* ranged from 0.010% to 0.207%. The obtained results provided useful information for quality assurance and standardization of *W. indica* in Vietnam.

Keywords: *Wikstroemia indica* (L.), *Aerial parts, HPLC-DAD, Daphnoretin.*

1. Introduction

Wikstroemia indica (WI) is a wild plant belonging to the Thymelaeaceae family. It is distributed in several countries, including Australia, China, India, Indonesia, Malaysia, Papua New Guinea, Philippines, Taiwan,

Thailand, and Vietnam [1],[2]. In Vietnam, *W. indica* is widely distributed in some provinces such as Bac Giang, Lang Son, Thanh Hoa (Sam Son), and Quang Tri. Traditionally, *WI* has been widely used to treat anemia, arthritis, cancer, syphilis, and whooping cough. Several investigations have demonstrated that *WI* has several pharmacological properties, such as anti-diarrheal, anti-inflammatory, and anti-microbial effects. Additionally, its cytotoxic activity has been assayed [2]. The antidiarrheal and thrombolytic effects of *WI* leaf extract have also been investigated [3].

WI contains abundant active components, including flavonoids, biflavonoids, coumarins, lignans, terpenoids, sterols, essential oils, and polysaccharides. One of the main components of *WI* is daphnoretin, which is a highly biologically active bis-coumarin with many biological activities, including antifungal effects, anti-cancer, anti-viral effects on hepatitis B, and respiratory syncytial virus properties [1]. Daphnoretin has been found in the roots, stems, fruits, and leaves of *WI* [4]. Latest edition (5^{th}) of the Vietnamese Pharmacopoeia does not include the monograph of *WI* [5]. However, the HongKong Chinese Materia Medica Standards (HKCMMS) [6] has a monograph for the medicinal roots of *WI* (L.), using reference standards daphnoretin in quantification assay, and the content of daphnoretin is specified as not less than 0.095%. In this study, we developed a HPLC-DAD method to determine the content of daphnoretin in the aerial parts of *WI* samples collected in Vietnam.

2. Materials and methods

2.1. Materials

The aerial parts of *WI* samples were collected in Autumn 2022 (September-October) from Bac Giang and Thanh Hoa Provinces. These samples were identified by Associate Professor Doctor Pham Thanh Huyen from National Institute of Medicinal. These samples were cleaned, dried in the oven at 60°C, and grounded into powder before extraction.

2.2. Chemicals

Daphnoretin was isolated from the aerial parts of *WI*. The structures of the compound were determined based on spectral data (MS, IR, NMR). The purity of daphnoretin was then determined by calculating the primary peak area as a percentage of total peak area by HPLC-DAD and the results suggested the purity of daphnoretin to be above 98%.

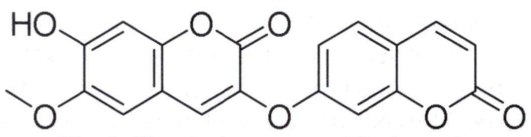


Fig. 1. Chemical structure of daphnoretin

Solvents for HPLC (methanol, acetonitrile) were from Merck - Germany. All other solvents and chemicals used for sample preparation (methanol, ethanol, ...) were of analytical grade (P.A.). Distilled water used is deionized double distilled water.

2.3. Instrumentation

The HPLC system (Shimadzu, Japan) used for the analysis was consisted of with a pump LC-20AD, a SIL-30AC autosampler, a CTO-10AS column oven and a diode-array detector (SPD-M20A). Quantitative estimation was performed with LabSolutions software programs. The separation of compounds was performed on an Agilent C_{18} column (250 x 4.6 mm, 5 µm). The mobile phase was a mixture of acetonitrile and water (40/60, v/v). The solvent flow rate was kept at 0.6 mL/min. The injection volume was 10 µL. Detection was set at a wavelength of 345 nm.

2.4. Methods

2.4.1. Optimization of chromatographic conditions:

- Detection wavelength (λ_{max}): the λ_{max} is selected based on UV-VIS spectrum of daphnoretin and combination with reference.
- Mobile phase and elution program: methanol - water and acetonitrile - water with different elution isocratic programs.

The optimized HPLC condition was established by comparing the resolution, retention time, asymmetry factor of analyzed peaks in each chromatogram after testing.

2.4.2. Optimization of the sample extraction:

Sample preparation factors were investigated including: Extraction solvents (ethanol, methanol, the mixture of ethanol-water and methanol-water with different ratios); Extraction methods (ultrasonic and heat reflux methods); Extraction time (30, 45, 60, 90 and 120 mins), ratio of the mass of the sample to the volume of the solvent (g/mL) (1/25, 1/50, 1/100) and number of times of extraction (1-3 times). Select the test sample preparation method to get the highest content of daphnoretin (greatest extraction efficiency).

2.4.3. Validation of the Method:

Analytical method validation was performed in accordance with ICH guidelines (and AOAC regulatory references), including assessments of:

Specificity, System suitability, calibration curves, limits of detection (LOD), limits of quantification (LOQ), precision, stability, repeatability and accuracy [7],[8].

2.4.4. Calculation formula:

The daphnoretin content in the test sample was calculated according to the following formula:

$$X\,(\%) = \frac{C \times V \times 100 \times K}{m \times 10^6 \times (100 - B)}$$

In which: X (%): the content of daphnoretin in test sample calculated with reference to the dried substance; C: the concentration of analyzed compound in the sample solution from the calibration curve equation (μg/mL); m: weight of tested sample taken to prepare the sample solution (g); B: water content of test sample (%); K: dilution factor.

3. Ressult and discussion

3.1. Optimization of HPLC conditions

To obtain accurate and optimal chromatographic conditions, different HPLC parameters were examined and compared, including detection wavelength, mobile phase, and elution program (methanol - water and acetonitrile - water with different elution programs).

Based on the maximum absorption of daphnoretin in the UV-VIS spectrum (Fig. 2) and combination with references [9], the detection wavelength was set at 345 nm for quantification of daphnoretin.

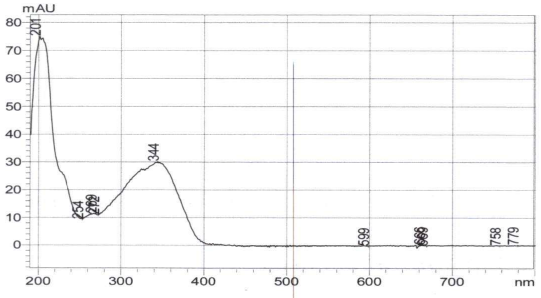


Fig. 2. UV spectrum of daphnoretin

The mobile phase conditions optimization is presented in Table 1.

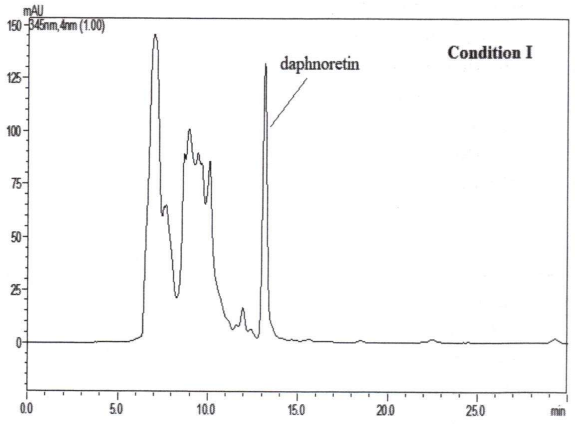
Table 1. Investigated mobile phase conditions.

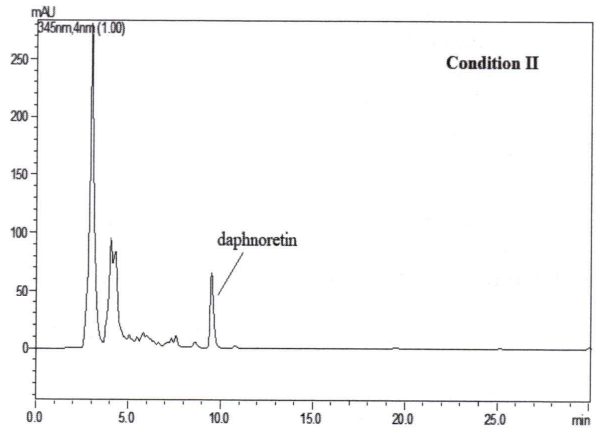
Conditions	Elution program
I	Methanol - Water (50:50), flow rate: 0.7 mL/min
II	Acetonitrile - 0.1% formic acid (30:70), flow rate: 0.7 mL/min (according to the (HKCMMS) [6])
III	Acetonitrile - Water (40:60), flow rate: 0.6 mL/min

Table 2. Chromatographic parameters of daphnoretin peaks under different conditions

Parameter	Retention time (min)	Symmetry factor ($0.8 \leq As \leq 1.5$)	Resolution ($Rs \geq 1.5$)	Number of theoretical plates
Condition I	13.12	1.459	0.867	13325
Condition II	9.48	1.472	2.203	11501
Condition III	11.97	1.140	2.553	11634

The results (Fig. 3 and Table 2) showed that condition III has the best separation efficiency within 30 minutes. Therefore, it was selected as the mobile phase program in our procedure.





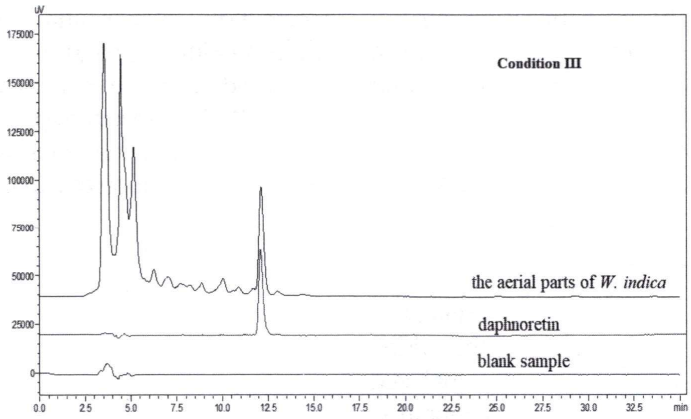


Fig. 3. Chromatograms for analysis of daphnoretin in the aerial parts of *WI* under different conditions

3.2. Calibration curve

The calibration curve was constructed by plotting the peak areas versus the concentrations of analyte. The result showed satisfactory linearity in the concentration ranges of 14.88 - 238.00 µg/mL for daphnoretin (Table 3).

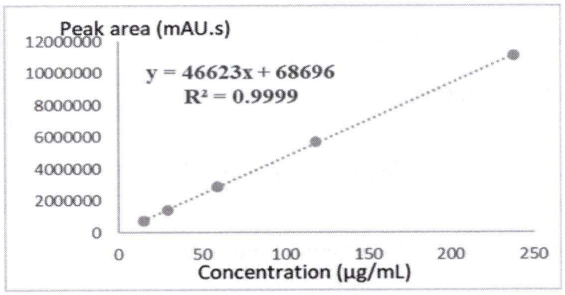


Fig. 4. Standard calibration curve of daphnoretin

Table 3. Linearity study data

Concentration (µg/mL)	Peak area (mAU.s)
238.00	11127131
119.00	5693846
59.50	2858988
29.75	*1437376*
14.88	725584

3.3. Optimization of extraction conditions

Methanol, ethanol and aqueous ethanol solutions were tested as extraction solvents. Several extraction conditions were fixed: m = 1 gram of sample, extraction solvent volume: 50 mL, extraction method: heat reflux, extraction time: 30 min. The extraction efficiency was evaluated through the daphnoretin content and showed in Table 4.

Table 4. The results of extraction conditions optimization

Extraction solvents *(m = 1 gram, solvent volume: 50 mL, extraction method: heat reflux, extraction time: 30 min)*		**Extraction methods and Extraction time** *(m = 1 gram, solvent volume: 50 mL of ethanol 80%)*	
Conditions	**Daphnoretin (%)**	**Conditions**	**Daphnoretin (%)**
Water	0.039	Sonication 15min	0.065
ethanol 50%	0.100	Sonication 30min	0.079
ethanol 60%	0.097	Sonication 45min	0.078
ethanol 70%	0.097	Sonication 60min	0.108
ethanol 80%	**0.104**	Sonication 90min	0.112
ethanol 90%	0.083	Reflux 30min	0.104
ethanol 100%	0.073	**Reflux 45min**	**0.111**
methanol 50%	0.063	Reflux 60min	0.112
methanol 70%	0.100	Reflux 90min	0.116
methanol 80%	0.100	Reflux 120min	0.110
methanol 90%	0.095		
methanol 100%	0.109		
Ratio of the sample mass to the solvent volume (g/mL) and number of extraction times (m = 1 gram, solvent: ethanol 80%, extraction method: heat reflux, extraction time: 45 min)			
Conditions	**Daphnoretin (%)**	**Conditions**	**Daphnoretin (%)**
1 gram/25 mL	0.101	1 gram/50 mL, 1 time	0.112
1 gram/50 mL	0.112	1 gram/50 mL, 2 times	0.111
1 gram/100 mL	0.113	1 gram/50 mL, 3 times	0.112

From the investigation results, the sample preparation procedure was selected as follows: Weigh accurately 1.0 g of the powdered sample and place it in a 100-mL round-bottomed flask, then add 50 mL of ethanol 80%. Reflux the mixture for 45 min. Cool down to room temperature. Transfer the solution to a 50-mL volumetric flask and make up to the mark with ethanol 80%. Filter through a 0.45-µm filter prior to HPLC analysis.

3.4. Method Validation

3.4.1. Selectivity:

The experiment was performed with a blank sample, daphnoretin and sample solutions according to the analytical procedure. The obtained chromatograms are shown in Fig. 3. The obtained results show that the peak signal of daphnoretin is sharp and well separated. Comparison of the UV spectrum of daphnoretin in the test sample and the standard sample showed that the matching ratio was above 0.99 (Fig. 5), indicating that the analytical method met the requirements for specificity.

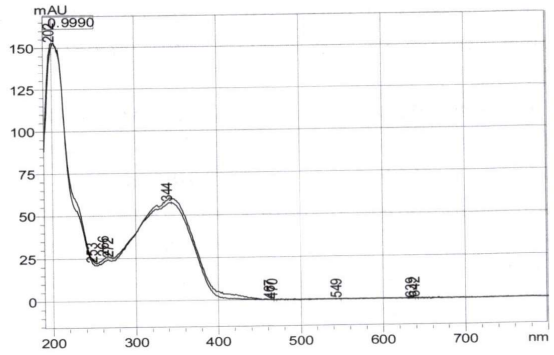


Fig. 5. UV spectrum overlay

3.4.2. System suitability:

Table 5 showed that RSD% of retention time and peak areas were all under 2%. These indicated that the developed method conforms system suitability criteria.

Table 5. System suitability testing (n = 6)

Parameter	Daphnoretin (29.75 µg/ml)	
	Mean	RSD (%)
Retention time, t_R (min)	11.97	0.15
Peak area (mAu.s)	1432485.80	0.21

3.4.3. Limit of Detection (LOD) and Limit of Quantification (LOQ):

The limits of detection (LOD) and the limits of quantification (LOQ) were calculated by diluting sample solutions until signal-to-noise ratios of 3 and 10, respectively. LOD and LOQ were estimated to be 0.11 µg/mL and 0.35 µg/mL for daphnoretin.

3.4.4. Precision:

The intra- and inter-day precisions have been summarized in Table 6. The RSD% values were all under 2%. These results showed that the method had good precision.

Table 6. Precision study by the proposed procedure

No.	Daphnoretin content (%)		
	Day 1	Day 2	
1	0.105	0.110	**Average**: 0.108 (%) **RSD**: 1.643%
2	0.109	0.107	
3	0.107	0.109	
4	0.106	0.109	
5	0.110	0.110	
6	0.106	0.110	
Average	**0.107**	**0.109**	
RSD%	**1.629**	**1.071**	

3.4.5. Accuracy:

The accuracy was evaluated by adding a known amount of daphnoretin at 3 different levels to the known sample and then extraction and analysis were done. The results in Table 7 showed that recovery rates of daphnoretin were in the range 95% - 105%. The results indicated that the accuracy of the method was acceptable.

Table 7. Recovery study of daphnoretin by the proposed procedure

TT	Amount spiked (µg/mL)	Amount found (µg/mL)	Recovery (%)
1	16.8	16.06	95.60
2	16.8	17.00	101.22
3	16.8	16.12	95.93
4	21.0	21.59	102.79
5	21.0	21.61	102.92
6	21.0	21.53	102.54
7	25.2	25.60	101.61
8	25.2	24.17	95.92
9	25.2	25.40	100.78
Mean			**99.92**
RSD%			**3.16**

3.5. Samples analysis

The proposed method was applied to determination of daphnoretin content in some samples of the aerial parts of *WI.* The results were presented in Table 8.

Table 8. The content of daphnoretin in the aerial parts of *WI* (n=3)

No	Sample name	Collected place of sample	Daphnoretin content Avg. ± SD (%)
1	M1	Bac Giang	0.108 ± 0.004
2	M2	Bac Giang	0.053 ± 0.003
3	M3	Bac Giang	0.207 ± 0.005
4	M4	Lang Son	0.141 ± 0.004
5	M5	Thanh Hoa	0.079 ± 0.003

3.6. Discussion

Wikstroemia indica (L.) C. A. Mey (Thymelaeaceae) has been widely used as a folk medicine for the treatment of syphilis, arthritis, whooping cough, and cancer. Due to its activities, this herbal medicine has an attractive potential as a promising natural agent in the pharmaceutical industry. However, the Vietnamese Pharmacopoeia has not been recognized as a monograph on *Wikstroemia indica*. Standardization of this medicinal plant is important and necessary. A proper method for the quantitative determination of daphnoretin - one of the main components - in the aerial parts of *WI* collected in Vietnam is required. HPLC-DAD is a convenient and accurate technical method for the qualitative and quantitative analysis of active components in herbal drugs.

Scientific research has confirmed the antifungal, anti-cancer, anti-viral effects on hepatitis B and respiratory syncytial virus properties of daphnoretin in *WI* [10],[11],[12]. The monograph *Wikstroemiae Radix* in HKCMMS [6] prescribed daphnoretin not less than 0.095% by HPLC-DAD. Ko Yen-Chen and his colleagues had confirmed that daphnoretin was found in the stem bark, stem wood, and fruit of *WI* by HPLC-PDA-MS [3]. In this study, the contents of daphnoretin in five samples of aerial parts of *WI* collected in some provinces in Vietnam varied from 0.053-0.207%. These results showed that daphnoretin meets the requirements for use as a marker according to the WHO guidelines [13] in the standardization of *WI*. The results of this study will be valuable the in standardization and quality evaluation of the aerial parts of *WI* collected in Vietnam.

4. Conclusions

Based on HPLC-DAD, we developed and validated a simple, reliable, and precise quantitative method for the analysis of daphnoretin in *Wikstroemia indica* (L.). The validation results displayed good accuracy, repeatability, linearity, and selectivity according to the ICH guideline. The obtained results provided useful information for the quality control of the *Wikstroemia indica* (L.) in Vietnam.

Acknowledgements: *The study was completed with financial support from the Vietnam-Korea Protocol "Study on anticancer and immunomodulatory activities of some Vietnamese medicinal plants, Investor.85.KR/20, Contract No. 09/2020/HD-NDT signed on 09/9/2020-Ministry of Science and Technology* ".

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