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ISOLATION AND PURIFICATION OF DARUTOSIDE FROM *SIEGESBECKIA ORIENTALIS* FOR ESTABLISHING A REFERENCE SUBSTANCE

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Summary

Isolation and Purification of Darutoside From *Siegesbeckia orientalis* for Establishing a Reference Substance

Siegesbeckia orientalis L. is an important medicinal herb that has been used traditionally in Vietnam, China for the treatment of back pain, knee pain, bone and joint pain, numb limbs, and boils. In this study, it is showed that *S. orientalis* collected in Thanh Hoa province, Vietnam contained significant content of darutoside, a reported constituent with various pharmacological activities. Darutoside was isolated and purified from the aerial parts of *S. orientalis* by using chromatography methods. The structure of the pure compound was identified by UV, IR, NMR, MS spectrometry. The establishment of a reference substance (RS) for darutoside was carried out according to ISO Guide 35 and WHO guideline. The purity of darutoside was determined as 95.16 ± 0.05% by HPLC. The results suggest that the isolated darutoside in this study could be used to be a RS for quality assessment of *S. orientalis* and related products.

Keywords: *Siegesbeckia orientalis, Darutoside, Reference substance.*

1. Introduction

The medicinal plant *Siegesbeckia orientalis* L. (family Asteraceae) has been traditionally used in Vietnam and China for the treatment of back pain, knee pain, bone and joint pain, numb limbs, and boils [1]. It has also widely been used in many pharmaceutical products in modern dosage forms [2].

Diterpenoids including *ent*-kauranes and *ent*-pimaranes have been reported as main chemical constituents in *S. orientalis* [3],[4]. Of which, kirenol and darutoside have been found to possess a variety of pharmacological activities such as topical anti-inflammatory and pain relieving, wound healing effects [5],[6]. Chinese [7] and Hongkong Pharmacopeias [8] regulate kirenol as the standard for the assay of *S. orientalis* by HPLC, meanwhile, Vietnamese Pharmacopoeia only tested for extractives in ethanol 96% [1]. Recent research showed that *S. orientalis* collected in Vietnam (Thanh Hoa, Nghe An...) contained not only kirenol but also a significant content of darutoside [2]. According to "WHO guidelines for selecting marker substances of herbal origin for quality control of herbal medicines", apart from meeting requirements for reference substance (RS), which isolated from herbs should be selected based on following priorities: (1) marker substances of constituents with known therapeutic activity; (2) marker substances of constituents with recognized pharmacological activities; (3) marker substances of characteristic constituents [9]. The previous reports indicated that darutoside is suitable to be selected as a marker substance for quality control of *S. orientalis* [2],[6]. This paper

presents the extraction, isolation of darutoside from *S. orientalis* and RS establishment for the isolated darutoside.

2. Materials and methods

2.1. Plant materials:

The herb *S. orientalis* was collected in Thach Thanh district, Thanh Hoa province in June 2020. The aerial parts were removed from the roots, cleaned, and dried at 40 - 50°C for 12 hours.

2.2. General experiment procedures

IR spectrum was recorded in KBr disc on a Bruker Tensor 27 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVIII-600 NMR instrument equipped with CryoProbe (Bruker, Karlsruhe, Germany) at 600 MHz (^{1}H) and 150 MHz (^{13}C). High-resolution electrospray ionization (HR-ESI-MS) spectrum was measured on an SCIEX X500 QTOF instrument. The purity of darutoside was determined on a Shimadzu CBM-20A HPLC system equipped with a LC-20AD pump, SPD-20A detector.

2.3. Extraction and isolation

Dried aerial parts of *S. orientalis* (20 kg) were extracted by maceration with ethanol 96% at room temperature for 14 days. The solvent was evaporated under reduced pressure to yield an ethanol extract (1.4 kg). The crude extract was then suspended in H_2O (3 L) and sequentially partitioned with *n*-hexane (6 L × 3 times), ethyl acetate (6 L × 3 times), *n*-BuOH (6 L × 3 times). These fractions were further analyzed by TLC, and the results showed that the diterpenoids were mainly in the ethyl acetate fraction. The EtOAc fraction (610 g) was chromatographed by using a *silica gel* column (63−200 μm particle size, 5 × 120 cm), eluting with a CH_2Cl_2-MeOH gradient system (100:1 to 0:1, each 5 L), to give 8 fractions (HTE1-HTE8) according to TLC analysis results. Fraction 5 (21.3 g) was subjected to RP-C18 column (3 × 80 cm), stepwise gradient of MeOH-H_2O (1:1 to 4:1) to afford 6 fractions (HTE5A-HTE5F). Fraction HTE5C (3.2 g) was subjected to RP-C18 column (3 × 80 cm) with mobile phase of MeOH-H_2O (2:1) to obtain compound **1** (610 mg). The isolation was respeated twice to yield 1 gram (1000 mg) of compound **1**.

2.4. RS establishment for the isolated darutoside

2.4.1. Impurity testing

+Test solution: the solution of 0.5 mg/ml of the isolated darutoside (**1**) in MeOH.

+ Impurity testing of the isolated darutoside (**1**) was performed by HPLC with three conditions as in Table 1.

+ The method was validated following ICH guideline [12].

Table 1. HPLC conditions for impurity testing of the isolated darutoside

Program No. 1 [8]			**Program No. 2 [7]**			**Program No. 3**		
- Column RP 18, 5μm (25 cm × 4.6 mm). - PDA detector at 215 nm - Mobile phase: + Phase A: acetonitrile + Phase B: phosphoric acid 0.2 % (v/v)			- Column RP 18, 5μm (25 cm × 4.6 mm). - PDA detector at 215 nm - Mobile phase: + Phase A: acetonitrile + Phase B: water			- Column RP 18, 5μm (25 cm × 4.6 mm). - PDA detector at 210 nm - Mobile phase: + Phase A: water + Phase B: acetonitrile		
Time (min)	Phase A (% v/v)	Phase B (% v/v)	Time (min)	Phase A (% v/v)	Phase B (% v/v)	Time (min)	Phase A (% v/v)	Phase B (% v/v)
0 - 20	27	73	0 - 5	5 - 24	95 - 76	0-35	80 - 50	20 - 50
20 - 60	27 - 40	73 - 60	5 - 80	24	76	35 - 60	50 - 0	50 - 100
- Flow rate: 1.0 mL/min			- Flow rate: 1.0 mL/min			- Flow rate: 1.0 mL/min		

2.4.2. Loss on drying and residue on ignition

Determination of loss on drying and residue on ignition of the isolated darutoside were conducted according to Vietnamese Pharmacopoeia (Appendixes 9.6 and 9.9, method 2).

2.4.3. Packaging, inter-laboratory evaluation and determination of assigned value and uncertainty of the reference substance:

Packaging, inter-laboratory evaluation and determination of assigned value and uncertainty of the reference substance were carried out according to ISO Guide 35 [10] and WHO Guideline [11].

3. Results and discussions

3.1. Structural elucidation

Compound 1: Amorphous powder; IR (KBr) ν_{max} 3398, 2938, 1647, 1473, 1079, 1028 cm^{-1}; HR-ESI-MS (*m/z*): 529.3026 $[M+HCOO]^-$; ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data, see Table 2.

The compound **1** was purified as an amorphous powder with the HR-ESI-MS data at *m/z* 529.3023 $[M+HCOO]^-$ (calcd. for $[C_{26}H_{44}O_8+HCOO]^-$ 529.3018) (Fig.1). The IR spectrum of **1** showed

absorption peaks for hydroxyl, alkene, alkane groups at 3398, 1647, and 2873 cm^{-1}, respectively (Fig. 2). The ^1H NMR of **1** showed an olefinic singlet proton signal at δ_H 5.18 (1H, br s, H-14), along with four signals of methyl singlet at δ_H 0.86 (3H, s, Me-17), 1.07 (3H, s, Me-18), 0.88 (3H, s, Me-19) and 0.86 (3H, s, Me-20). The ^{13}C NMR of compound **1** displayed 26 signals, of which 6 carbons at δ_C 101.9 (C-1'), 75.2 (C-2'), 78.3 (C-3'), 71.9 (C-4'), 77.7 (C-5') and 63.0 (C-6') were assigned to a glucopyranose moiety. An anomeric proton at δ_H 4.34 (1H, d, J = 7.8 Hz, H-1') suggested the presence of a β-D-configuration [13]. In addition, the signals of two olefinic carbons (δ_C 139.9 and 129.5) and three oxygenated carbons (δ_C 86.0, 77.5 and 64.3) were observed in the ^{13}C NMR spectrum of **1**. Based on the above evidence and comparison with the spectroscopic data in literature (Table 2), compound **1** was identified as darutoside [13].

Table 2. ^1H (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data of **1** and darutoside

TT	**1**[a]		**Darutoside [13]**[a,b]	
	^1H (J in Hz)	^{13}C	^1H (J in Hz)	^{13}C
1a	1.76 m	38.1	1.76 dt (13.0, 3.9)	38.0
1b	1.18 td (13.2, 3.6)		1.15 td (13.0, 3.9)	
2a	1.57 m	24.4	1.56 m	24.3
2b	1.82 m		1.80 m	
3	3.39 m	86.0	3.37 m	86.0
4	-	39.4	-	39.3
5	1.12 dd (13.2, 2.4)	56.2	1.10 dd (13.0, 2.2)	56.1
6a	1.45 m	23.4	1.38 dd (13.0, 2.2)	23.3
6b	1.65 m		1.63 m	
7a	2.33 ddd (14.2, 4.5, 2.0)	37.1	2.27 m	37.0
7b	2.06 td (14.4, 5.4)		2.03 td (14.0, 5.6)	
8	-	139.9	-	139.7
9	1.71 m	52.1	1.70 m	52.0
10	-	39.0	-	38.9
11a	1.59 m	19.4	1.52 m	19.3
11b	1.59 m		1.52 m	
12a	2.00 m	33.3	1.97 m	33.2
12b	0.94 m		0.92 m	
13	-	38.5	-	38.4
14	5.18 br s	129.5	5.15 br s	129.4
15	3.59 dd (9.0, 2.4)	77.5	3.57 dd (8.8, 1.5)	77.4
16a	3.48 dd (11.4, 9.0)	64.3	3.46 dd (10.5, 8.8)	64.2
16b	3.71 dd (11.4, 2.4)		3.69 dd (10.5, 1.5)	
17	0.86 s	23.1	0.83 s	23.0
18	1.07 s	29.2	1.04 s	29.1
19	0.88 s	17.3	0.82 s	17.3
20	0.86 s	15.3	0.85 s	15.3
1'	4.34 d (7.8)	101.9	4.32 d (7.8)	101.8
2'	3.19 dd (9.0, 7.8)	75.2	3.17 dd (8.9, 7.8)	75.0
3'	3.38 t (9.0)	78.3	3.36 t (8.9)	78.1
4'	3.32 t (9.0)	71.9	3.31 t (8.9)	71.8
5'	3.26 m	77.7	3.24 m	77.5
6'a	3.69 dd (11.4, 5.4)	63.0	3.68 dd (12.3, 5.3)	62.9
6'b	3.87 dd (11.4, 2.4)		3.85 dd (12.3, 1.8)	

[a]recorded in CD3OD; [b]reported in literature [13].

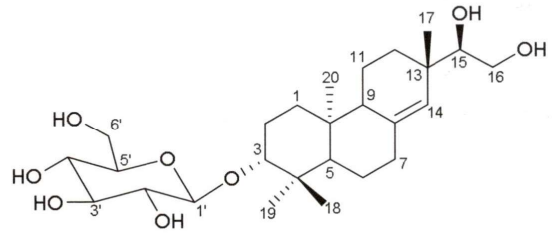


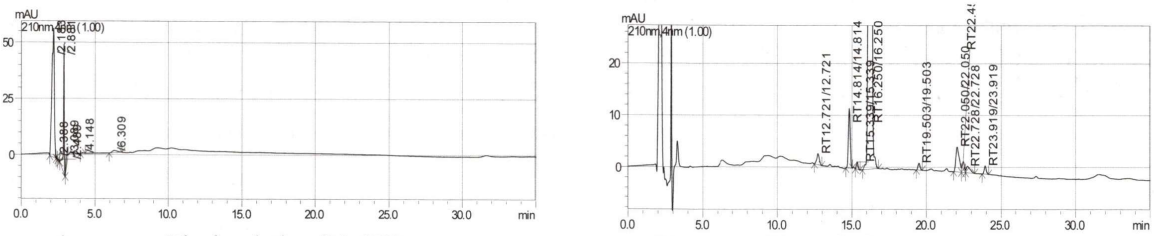
Fig. 1. Chemical structure of **1**

3.2. Establishment of RS for isolated darutoside

3.2.1. Determination of related substances (organic impurities) in the isolated darutoside:

Selection of optimal HPLC condition: Among three HPLC conditions (Table 1), all peaks including one main peak and 8 peaks of related substances in Program No. 3 were well separated,

the purity index of the main peak was 0.999998. The retention times were 15.33 min for daurutoside and 12.72, 14.81, 16.25, 19.50, 22.05, 22.45, 22.72, 23.92 min for the related substances. Thus, program No. 3 was chosen for determination of the related substances in the isolated darutoside (Fig. 2). The selected condition was validated for specificity in stress conditions, linearity, LOD, LOQ, repeatability, reproducibility. The validation results were summarized in Table 3.



A. Blank solution (MeOH) B. Test solution

Fig. 2. HPLC chromatogram for impurity testing of the isolated darutoside

Table 3. Summary of validation results

Characteristic	Results
Specificity	- Chromatogram of blank solution did not show any peak at retention time of main peak and the other 8 related substances peaks - Chromatograms of test solution under stress condition (UV, temperature, NaOH, HCl and H_2O_2) had well separated peaks including darutoside peak and other 8 related substance peaks
LOD, LOQ	LOD = 0.05 µg/ml; LOQ = 0.10 µg/ml
Linearity	0.10 µg/ml-843.30 µg/ml; R^2 = 0.9997; analysis of linear regression showed p-value for intercept = 0.8796 > 0.05
Repeatability of total related substances	RSD = 1.33%, total related substances = 3.11%
Reproducibility of total related substances	RSD = 1.95%, total related substances = 3.08%

3.2.2. Determination of loss on drying and residue on ignition of the isolated darutoside:

- Content (purity) of darutoside RS was calculated following mass-balance formula:

X(%) = 100*[(100 - %HPLC Related substances)/100]*{[100 - (% Loss on drying + % Residue on ignition)]/100}

- Assay of loss on drying was applied to measure the total amount of water and volatile impurities while Residue on ignition was performed to determine non-volatile inorganic impurities. The results showed that the loss on drying and residue on ignition of the isolated darutoside were 1.82% (u=0.01%) and 0.04% (u=3.10^{-7}%), respectively.

3.2.3. Packaging and evaluation of the batch homogeneity

The isolated darutoside was packed in 2 ml brown glass vials (100 vials) in Glove box Labconco in one day, under controlled condition (25.3 - 26.2°C; N_2 purity ≈ 99,999 %). Each vial contained 10 mg of darutoside.

The vials were numbered. Ten vials were randomly selected for homogeneity test. From each vial, weighed accurately 5 mg of darutoside, dissolved in 10 ml of MeOH, performed the above selected HPLC program and recorded areas of the main peak. The first sample was assumed to contain 100% of darutoside. The contents of darutoside in nine other samples were calculated on the basis of peak area compared with that of the first sample. The results were summarized in Table 4.

Table 4. The results of batch homogeneity

Vial No.	Weight (mg)	Peak area (mAU.s)	Content (%)
1	4.957	8433838	100.00
8	5.106	8574728	98.70
20	5.035	8554357	98.68
31	5.367	9122267	99.90
40	4.938	8292056	98.70

Vial No.	Weight (mg)	Peak area (mAU.s)	Content (%)
51	4.946	8341938	99.13
62	4.972	8447091	99.85
75	5.126	8666898	98.23
82	5.090	8610103	98.47
90	5.001	8511195	100.21
Average			**99.50** RSD = 0.52 %, n=10

The RSD value was 0.52 (< 1,0 %) (Table 4) indicating the batch uniformity of darutoside RS.

3.2.3. Inter-laboratory evaluation of darutoside RS

The darutoside RS vials were randomly selected and quantified the total related substances (peak area percentage) in three independent laboratories belong to National Institute of Drug Quality Control (Lab. for establishment of reference substance, Lab. for research and development, Lab. for physical measurements). Six vials were quantified at each laboratory. The results obtained from 3 laboratories were summarized in Table 5.

Table 5. Related substances content determined in three independent laboratories

No.	Total related substances (%)		
	Lab. No. 1	***Lab. No.2***	***Lab. No.3***
1.	2.94	2.95	3.16
2.	2.96	3.06	3.08
3.	3.07	2.92	3.05
4.	3.10	2.98	3.14
5.	3.11	3.11	2.97
6.	3.09	2.95	3.10
Average (%)	3.04	3.00	3.08
RSD (%)	2.34	2.49	2.13
Uncertainty (type A) u = s/√n	0.030	0.030	0.0267
Combined of 3 laboratories (n=18)	**Average = 3.04 %** s = 0.08; RSD = 2.51 %		

+ RSD (%) of repeatability for each laboratory and RSD (%) of reproducibility from 3 laboratories < 5%, so the repeatability and reproducibility meet the ICH requirement at this tested concentration.

+ Anova analysis showed $F_{tn} = 2.13 < F_{tc} = 3.68$, so the results from 3 laboratories were not significant different. In addition, the 3 laboratories are competent laboratories, so we used average arithmetic value as combined value for total related substances.

+ Uncertainty of related substance value can be calculated based on standard deviation of mean values of total related substances submitted by each laboratory (not using laboratory uncertainty statement). In this case:

u = s/√n = 0.025 (s: standard deviation of 3 mean values submitted by 3 laboratories, n = 3);

Or by combining uncertainties of 3 laboratories using the law of propagation:

$$u = \frac{1}{3}\sqrt{u_{lab.1}^2 + u_{lab.2}^2 + u_{lab.3}^2} = 0.017$$

Compare these two results, we selected u = 0.025 as uncertainty of the mean value of total related substances (3.04%).

+ Assigned value of content of darutoride in established RS:

$X_{(\%)}$ = *[(100 - 3.04)/100]*[(100 - 0.04-1.82)/100]*100 = 95.16%*

+ Uncertainty of assigned value:

$$u_{RS} = \sqrt{u_{loss\ on\ drying}^2 + u_{residue\ on\ ignition}^2 + u_{related\ substances}^2} = 0.027$$

Expanded uncertainty at prohibition 95%: $U_{RS} = 2*u_{RS} = 0.05$

90 vials of darutoside RS (batch No. E0121382.01) (purity of 95.16 ± 0.05%) were produced for quantitative application.

4. Conclusions

One gram of darutoside was purified from the aerial parts of *S. orientalis* collected in Thanh Hoa province, Vietnam. The RS establishment for the isolated darutoside was conducted according to ISO Guide 35 and WHO guideline. 90 vials of darutoside RS (10 mg) (batch No. E0121382.01) were produced with the purity 95.16 ± 0.05%. Darutoside RS in this study could be used for the quality control of *S. orientalis* and pharmaceutical products from *S. orientalis*.

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FLAVONOIDS FROM THE ROOT OF *MILLETTIA PULCHRA* KURZ COLLECTED IN AN GIANG PROVINCE

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Summary

Flavonoids from the Root of *Millettia pulchra* Kurz Collected in An Giang Province

Millettia pulchra Kurz (Fabaceae) was called as "Bạch chỉ nam" and it has been used in the traditional medicine in Vietnam for the treatment of skin allergy, rheumatism bone pain, digestive disorder, abnormal pain, and diarrhoea. Seven compounds were isolated and identified as (6a*R**,11a*R**)-maackiain (**1**), daidzein (**2**), genistein (**3**), 7,5′-dihydroxy-3′-methoxyisoflavone (**4**), pseudobaptigenin (**5**), 3-*O*-methylbutein (**6**), and pongamol (**7**) from the ethyl acetate fraction from the root of *Millettia pulchra* Kurz. Their structures were determined by the mean of spectroscopic methods (MS, 1D and 2D NMR).

Keywords: *Millettia pulchra, Flavonoid, Isoflavonoid, Chalcone.*

1. Introduction

Millettia pulchra Kurz is small shrubs belonging to the Fabaceae family. There are 150 species of the genus *Millettia* which are distributed in the tropical and sub-tropical area in the Indian region and Southeast Asian countries. In Vietnam, there are 25 species widely distributed from the North to the South [1]. The main components from the root of *M. pulchra* were flavonoids, especially isoflavonoids, phenolics, sterols [2], and alkaloids [3]. The root of "Bạch chỉ nam" originated from *M. pulchra* Kurz has frequently been used in the oriental medicine in An Giang province for the treatment of skin allergy, rheumatism bone pain, digestive disorder, abnormal pain, and diarrhoea [1]. Our previous research on the root of *M. pulchra* let to the isolation of four compounds [4]. In our continuing study, seven flavonoids were isolated from the ethyl acetate fraction from the root of *M. pulchra*. Their structures were elucidated by spectroscopic data and compared to those of reported in the literature. In the present paper daidzein (**2**), 7,5′-dihydroxy-3′-methoxyisoflavone (**4**), pseudobaptigenin (**5**), and 3-*O*-methylbutein (**6**) are first reported in *Millettia pulchra*.

2. Materials and methods

2.1. Materials

The fresh root of *Millettia pulchra* Kurz (15 kg) was bought in An Giang province in November, 2018. The voucher specimen (MP-112018) was identified by Dr. Vo Van Chi and