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ESTABLISHMENT OF AN IN-HOUSE STANDARD FOR *PRUNELLAE SPICA*

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Summary

This study aimed to establish an in-house quality standard for *Prunellae spica* (PS) based on the Vietnamese Pharmacopoeia V, incorporating enhanced qualitative and quantitative specifications. The evaluated criteria included description, powder, water content, total ash, acid-insoluble ash, identification by chemical reaction and thin-layer chromatography, quantification of rosmarinic acid using high-performance liquid chromatography with diode array detection according to the Chinese Pharmacopoeia (2020), extractives, and foreign matter. Quantitative analysis of eight PS samples revealed that the rosmarinic acid content ranged from 0.172% to 0.860%, calculated with reference to the dried drug. The developed in-house standard was subsequently applied for the quality assessment of PS samples collected from the market.

Keywords: *In-house standard; Prunellae spica; Qualitative and quantitative analysis; HPLC-ĐA; Rosmarinic acid.*

1. Introduction

Prunellae spica (PS) is the dried fruiting spike (infructescence) of *Prunella vulgaris* L. (Lamiaceae) [1]. The species thrives in bright and humid environments, typically forming small, localized clusters with limited overall biomass. It can be propagated by seed, with flowering occurring approximately 75–90 days after cultivation. The blooming season extends from April to June, followed by fruiting from July to October. *P. vulgaris* L. is native to temperate regions across Europe and Asia, with a distribution covering China, Japan, India, and several European countries. In Vietnam, this species is found in cool, moist mountainous regions such as Sa Pa (Lao Cai Province), Tam Dao (Phu Tho Province), Mau Son (Lang Son Province), as well as in other provinces, such as Tuyen Quang, Lai Chau, and Quang Ngai. Traditionally, *P. vulgaris* L. has been used in folk medicine to treat various diseases such as epiphora, scrofula, goiter, mastitis, neurodermatitis, furuncles, tinea corporis, and psoriasis [2].

P. vulgaris L. contains abundant active components, including triterpenoids, sterols, phenolics, flavonoids, coumarins, phenylpropanoids, polysaccharides, and volatile oils [3]. In the Vietnamese Pharmacopoeia V (VP V), the monograph for PS includes criteria description, powder, water content, total ash, acid-insoluble ash, extractives, foreign matter, and identification by chemical reaction and thin-layer chromatography (TLC) using reference herbal material. However, quantitative assays have not yet been established. Among its phytochemical constituents, rosmarinic acid (RA) and ursolic acid (UA) are considered major bioactive compounds with diverse pharmacological effects. Numerous studies have demonstrated that RA exhibits anti-inflammatory, antioxidant, antidiabetic, antiviral, antitumor, neuroprotective, and hepatoprotective properties [4]. Likewise, UA possesses anti-inflammatory, antioxidant, anti-apoptotic, and anticancer activities [5]. In the Chinese Pharmacopoeia (CP) [6] and the Hong Kong Chinese Materia Medica Standards (HKCMMS) [7], RA is employed as a quality control marker for PS, whereas UA is used for the same purpose in the European Pharmacopoeia (EP) [8]. Therefore, this study was conducted to upgrade an in-house standard for PS according to the VP V and CP in terms of qualitative and quantitative specifications.

2. Materials and methods

2.1. Materials

Samples of *Prunellae spica* were collected from several provinces in Vietnam (Table 1) and identified based on morphological characteristics by Assoc. Prof. Pham Thanh Huyen, National Institute of Medicinal Materials. The plant materials were then oven-dried at 50°C and ground into powder before extraction.

Table 1. List of the test samples

No.	Samples	Location	Collection time
1	M1	SaPa, Lao Cai*	06.2025
2	M2	Quan Ba, Ha Giang	06.2025
3	M3	Dong Van, Ha Giang	07.2025
4	M4	Sin Ho, Lai Chau	07.2025
5	M5	Dong Van, Ha Giang	07.2025
6	M6	SaPa, Lao Cai	07.2025
7	M7	Quan Ba, Ha Giang	07.2025
8	M8	Quan Ba, Ha Giang	07.2025
9	TT1	Lan Ong Street, Hanoi	08.2025
10	TT2	Lan Ong Street, Hanoi	08.2025
11	TT3	Lan Ong Street, Hanoi	08.2025
12	TT4	Lan Ong Street, Hanoi	08.2025

*Samples M1-M8 were used to upgrade an in-house standard for PS; * Sample M1 was used for HPLC method validation; Samples TT1-TT4 were collected on the market.*

2.2. Chemicals

- Reference substances: Rosmarinic acid (CAS No. 20283-92-5; Lot No. CNF99103; purity 98%) and ursolic acid (CAS No. 77-52-1; Lot No. CNF97259; purity 98%) were purchased from ChemFaces (China).

- Reference medicinal materials: PS (Lot No. HP0125120) was obtained from the National Institute of Drug Quality Control (Vietnam).

- Solvents and reagents: Solvents and chemicals used for TLC and HPLC were purchased from Merck (Germany). All solvents and reagents used for extraction and sample preparation in quantitative analysis were of analytical grade. TLC $F_{254}S$ *silica gel* plates (Merck) were used for chromatographic analysis.

2.3. Instruments

The HPLC system (Shimadzu, Japan) used for analysis consisted of an LC-20AD pump, a SIL-20AHT autosampler, and a DAD detector. Data acquisition and processing were performed using LabSolutions software. The chromatographic separation was carried out on an Agilent C_{18} column (250 × 4.6 mm, 5 µm).

2.4. Methods

2.4.1. Description, powder, water content, total ash, acid-insoluble ash, extractives, foreign matter:

These criteria were determined in accordance with the procedures described in the VP V.

2.4.2. Identification:

a. Identification by chemical reaction: Performed according to the PS monograph in the VP V.

b. Identification by TLC:

- Test solution: Accurately weigh approximately 2.5 g of PS powder, add 30 mL of 70% ethanol, sonicate for 30 minutes, then filter. Concentrate the filtrate to dryness, and dissolve the residue in 5 mL of ethanol to obtain the solution.

- Reference medicinal material solution: Accurately weigh approximately 2.5 g of PS reference material powder and prepare in the same manner as the test solution.

- Reference substance solution (1): Dissolve rosmarinic acid reference standard in methanol to obtain a concentration of 0.1 mg/mL.

- Reference substance solution (2): Dissolve ursolic acid reference standard in methanol to obtain a concentration of 0.5 mg/mL.

- Chromatographic conditions:

Stationary phase: Pre-coated *silica gel* 60 GF_{254} TLC plates (Merck), activated at 100°C for 30 minutes.

Mobile phase (MP):

+ Condition I: cyclohexane – chloroform – ethyl acetate – glacial acetic acid (20:5:8:0.5, *v/v/v/v*)

+ Condition II: cyclohexane – ethyl acetate – isopropanol – formic acid (15:3:3.5:0.5, *v/v/v/v*)

+ Condition III: *n*-hexane – ethyl acetate – isopropanol – formic acid (8:3:1.25:0.1, *v/v/v/v*).

+ Condition IV: toluene – ethyl acetate – acetone – formic acid (5:2:1:0.75, *v/v/v/v*).

Detecting reagent: 10% sulfuric acid in ethanol.

Procedure: Spot 5 µL of the test solution and each reference solution separately on the TLC plate. Develop the chromatogram according to the VP V, Appendix 5.4. After the solvent front has migrated approximately 8 cm, remove the plate and allow it to dry at room temperature. Then, spray with the detecting reagent, heat at 120°C until clear spots appear, and observe under visible light.

2.4.3. Quantification of rosmarinic acid:

The content of rosmarinic acid in PS samples was determined by HPLC according to the PS monograph in the CP (2020), with minor modifications to suit the laboratory conditions.

➢ Chromatographic conditions:

- Column: C_{18} (250 × 4.6 mm, 5 μm)
- Flow rate: 1 mL/min
- Injection volume: 10 μL
- Detection wavelength: 330 nm
- Mobile phase: methanol – 0.1% trifluoroacetic acid (42:58, *v/v*)

➢ Preparation of solutions:

Test solution: Accurately weigh about 0.2500 g of PS powder into a stoppered conical flask. Add exactly 50 mL of 50% ethanol, weigh the flask, sonicate for 30 minutes, allow to cool, and reweigh. Compensate for any weight loss with 50% ethanol, mix well, and filter through a 0.45 μm membrane filter to obtain the test solution.

Standard solution: Accurately weigh an appropriate amount of rosmarinic acid reference standard and dissolve in methanol to prepare standard solutions with concentrations ranging from approximately 4 μg/mL to 500 μg/mL.

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

$$X\,(\%) = C \times \frac{50}{1000} \times \frac{100}{100 - h} \times \frac{100}{m}$$

In which: X (%): the content of rosmarinic acid 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 the tested sample taken to prepare the sample solution (g); h: water content of test sample (%).

3. Results and Discussion

3.1. Identification

3.1.1. Identification by chemical reaction:

All samples gave positive results in the reactions described in Section 2.4.2a.

3.1.2. Identification by TLC:

After performing the procedure described in Section 2.4.2b, the obtained chromatograms are shown in Fig. 1.

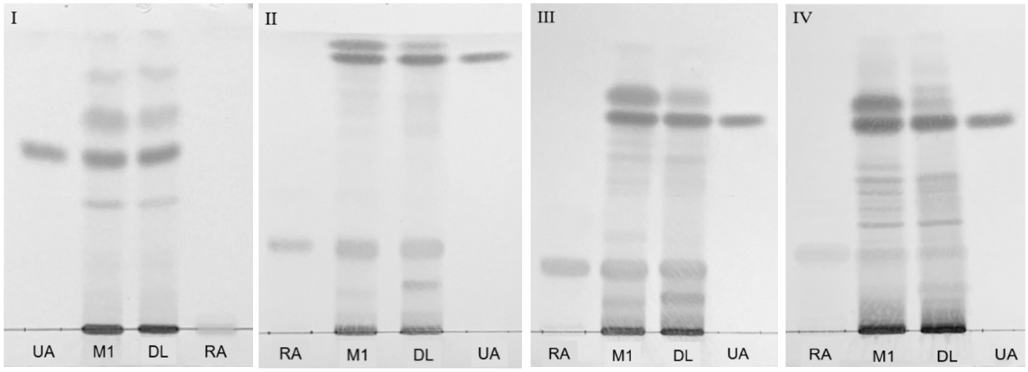

Fig. 1. Results of the investigation of TLC conditions
(UA: ursolic acid; RA: rosmarinic acid; M1: sample M1; DL: reference medicinal material)

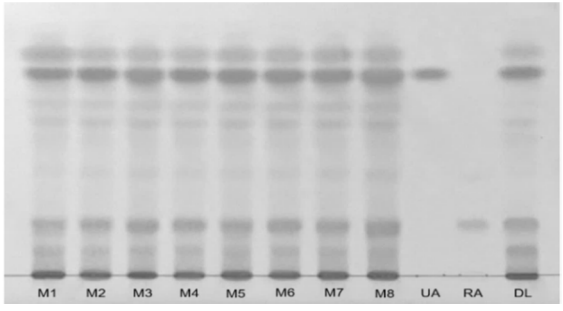

Fig. 2. TLC chromatograms for the identification of PS samples (M1–M8)
UA: ursolic acid; RA: rosmarinic acid; M1-M8: sample M1-M8; DL: PS *reference medicinal material*

Condition III was selected as the optimal mobile phase for the identification of RA and UA in PS samples. This condition allows simultaneous detection of both compounds, providing R_f values within the suitable range (RA: R_f = 0.2; UA: R_f = 0.8). The chromatographic spots are compact, well-resolved, and produced using solvents of low toxicity. The chromatograms of the test solutions exhibit spots with the same R_f values and colors as those of the corresponding reference solutions (Fig. 2).

3.2. Quantification of rosmarinic acid by HPLC

The HPLC method was validated following AOAC guidelines [9].

3.2.1. Method validation:

a) System suitability:

System suitability was verified by making six consecutive injections of the reference substance solutions. RSD (%) of retention time and peak areas were all under 2% (Table 2). These results indicate that to system suitability criteria.

Table 2. System suitability testing (n=6)

Parameter	RA (65.5 µg/mL)	
	Mean	RSD (%)
Retention time, t_R (min)	13.814	0.23
Peak area (mAu.s)	1804058	0.75

b) Selectivity:

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

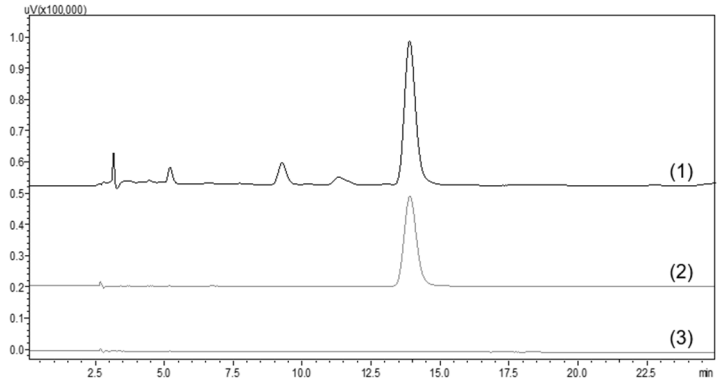

Fig. 3. Chromatograms for analysis of RA in PS under different conditions *(1): test sample; (2): rosmarinic acid; (3): blank sample*

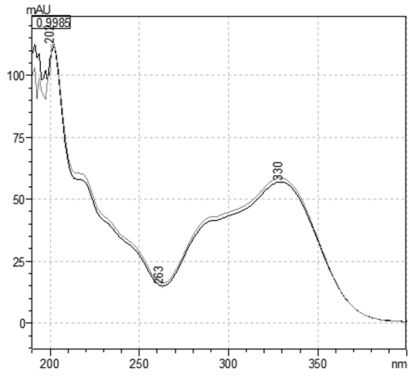

Fig. 4. UV spectrum overlay

c) Calibration curve

The calibration curve was constructed by plotting the peak areas versus the concentrations of the analyte. The result showed satisfactory linearity in the concentration ranges of 4.09 – 524.00 µg/mL for RA (Fig. 5 and Table 3). The calibration curve has $R^2 > 0.99$ and bias < 15%, indicating that there is a good linear correlation between the concentration of rosmarinic acid and the peak area. Therefore, the proposed method can be used for the quantitative analysis of RA.

Table 3. Linearity study data

Concentration (*µg*/mL)	Peak area (mAU.s)	Deviation (%)
524.00	14897264	0.09
262.00	7422351	-0.02
131.00	3648914	-1.20
65.50	1807457	-1.14
32.75	898369	0.23
16.38	440968	2.36
8.19	220149	9.99
4.09	97741	14.97

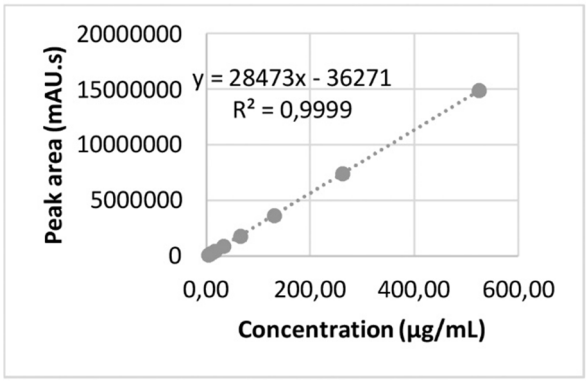

Fig. 5. Calibration curve of RA

d) Precision:

Table 4. Precision study by the proposed procedure

No.	Rosmarinic acid content (%)		
	Day 1	Day 2	
1	0.253	0.254	
2	0.258	0.263	
3	0.256	0.266	**Average:** 0.258%
4	0.262	0.255	
5	0.254	0.259	**RSD:** 2.106%
6	0.254	0.257	
Average	**0.256**	**0.259**	
RSD (%)	**1.219**	**1.624**	

The intra- and inter-day precisions have been summarized in Table 4. The RSD (%) value of intra-day was less than 2% and the RSD (%) value of inter-day was under 4%. These results showed that the method had good precision (the analyte content is in the range of 0.1% – 1%).

e) Accuracy

The accuracy was evaluated by adding a known amount of rosmarinic acid at 3 different levels (50%, 100%, 150%) to the known sample and then extraction and analysis were done.

Recovery (%) = 100 × Amount found/(Amount spiked + Initial amount).

In which: Initial amount: the quantity of RA originally present in the unspiked sample (mg); Amount spike: the known quantity of RA added to the sample (mg); Amount found: the experimentally determined quantity of RA (mg).

The results showed that recovery rates of rosmarinic acid were in the range 92% - 105%. The results indicated that the accuracy of the method was acceptable.

Table 5. Recovery study of rosmarinic acid by the proposed procedure

No.	Initial amount (mg)	Amount spiked (mg)	Amount found (mg)	Recovery (%)
1	0.552	0.262	0.799	98.26
2	0.552	0.262	0.797	97.95
3	0.552	0.262	0.796	97.84
4	0.557	0.524	1.059	97.98
5	0.557	0.524	1.062	98.26
6	0.557	0.524	1.073	99.32
7	0.546	0.786	1.279	96.01
8	0.546	0.786	1.267	95.13
9	0.546	0.786	1.279	96.03
Mean				**97.42**
RSD (%)				**1.32**

f) Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ values were determined by LOD was defined as a signal-to-noise ratio of 3:1 and a LOQ 9-10 times the level of noise in the samples. LOD and LOQ were estimated to be 0.22 μg/mL and 0.73 μg/mL for RA.

3.2.2. Sample analysis

The quantification results are presented in Table 6.

Table 6. The content of RA in the PS samples (n = 3)

No.	Samples	RA content (Avg. ± SD) (%)
1	M1	0.860 ± 0.022
2	M2	0.391 ± 0.008
3	M3	0.172 ± 0.006
4	M4	0.216 ± 0.006
5	M5	0.266 ± 0.002
6	M6	0.269 ± 0.008
7	M7	0.188 ± 0.007
8	M8	0.254 ± 0.002

No.	Samples	RA content (Avg. ± SD) (%)
VP V		-
CP (2020)		≥ 0.20
HKCMMS		≥ 0.041
Proposal		**≥ 0.20**

Quantitative analysis showed that the RA content in PS samples collected from different regions of Vietnam varied notably, ranging from 0.172 ± 0.006% (Dong Van, Ha Giang) to 0.860 ± 0.022% (Sa Pa, Lao Cai). According to the CP (2020), the RA content in PS should not be less than 0.20%, whereas the HKCMMS set a much lower limit (≥ 0.041%). Both pharmacopoeias employ the HPLC-DAD method using a mobile phase composed of methanol and 0.1% trifluoroacetic acid. The variation in RA content among samples may be attributed to differences in ecological conditions and geographic origins. As the PS monograph in the VP V does not specify a quantitative requirement for RA, we propose that the RA content in PS should not be less than 0.20% according to the CP (2020), calculated with reference to the dried drug.

3.3. Proposal for an in-house Standard for Prunellae spica

The upgrading of the in-house standard for *Prunellae spica* based on the Vietnamese Pharmacopoeia V, with improvements in both qualitative and quantitative specifications, is shown in Table 7.

Table 7. Proposal for an in-house Standard for *Prunellae spica*

Criteria	VP V	CP (2020)	HKCMMS	Results	Proposal
Description	✓	✓	✓	Cylindrical spikes, 1.5–8 cm long and 0.8–1.5 cm in diameter, are light brown to reddish brown in color. Each spike consists of more than ten calyces and bracts. Each whorl bears two opposite, fan-shaped bracts with an acuminate apex and distinct vein striations, densely covered with white hairs on the outer surface. Each bract contains three small flowers, whose corollas often fall off. The calyx is bilabiate and encloses four small, brown, ovoid nutlets with a white, convex area at the acute end. Texture light and brittle; odor slight; taste weak	The medicinal material must exhibit the characteristics as described
Powder	✓	✓	✓	Dark brown powder; odor slight; taste weak. Multicellular trichomes (5–7 cells), sometimes very long, with a dotted surface. Fragments of the lower epidermis of the leaf show stomata. Epidermal cells of the calyx have sinuous walls. Fragments of branch parenchyma consist of rectangular cells arranged alternately. Calcium oxalate crystals are spherical with blunt spines. Scalariform, spiral, and reticulate vessels are present. The stigma consists of elongated cells radiating like fan spokes	The medicinal material powder must exhibit the characteristics as described
Water content (%)	≤ 12.0	≤ 14.0	≤ 15.0	7.8 ± 0.2 – 10.9 ± 0.2	≤ 12.0
Total ash (%)	≤ 13.0	≤ 12.0	≤ 10.0	5.6 ± 0.1 – 8.5 ± 0.1	≤ 13.0
Acid-insoluble ash (%)	≤ 3.0	≤ 4.0	≤ 1.5	0.8 ± 0.1 – 2.2 ± 0.1	≤ 3.0
Identification by chemical reaction				Positive in the qualitative test	The medicinal material must show positive reactions in the qualitative tests
Identification by TLC	PS reference medicinal material	RA	UA	The chromatogram of the test solution exhibits spots with the same R_f values and colors as those of the reference solutions (PS reference medicinal material, RA, UA)	The chromatogram of the test solution must exhibit spots with the same R_f values and colors as those of the reference solutions

Criteria		VP V	CP (2020)	HKCMMS	Results	Proposal
Quantification – RA content (%)		×	≥ 0.20	≥ 0.041	0.172 ± 0.006 – 0.860 ± 0.022	≥ 0.20
Extractives (%)		≥ 7.0 (96% ethanol)	≥ 10.0 (water)	≥ 10.0 (water); ≥ 6.0 (96% ethanol)	7.2 ± 0.2 – 11.8 ± 0.1 (96% ethanol)	≥ 7.0 (96% ethanol)
Foreign matter (%)	Stems	≤ 5.0	≤ 5.0	≤ 1.0	1.2 ± 0.1– 3.5 ± 0.1	≤ 5.0
	Other	≤ 1.0	≤ 1.0	×	0.3 ± 0.2 – 0.8 ± 0.2	≤ 1.0

✓*criterion available,* × *criterion not available.*

3.4. Utilizing the developed in-house standard for quality assessment of PS samples collected on the market

The developed in-house standard was applied to evaluate the quality of several PS samples (TT1-TT4) collected on the market, and the results are presented in Table 8.

Table 8. Quality assessment results of several PS samples on the market

Samples	Water content (%)	Total ash (%)	Acid-insoluble ash (%)	Identification
TT1	7.2 ± 0.1	8.5 ± 0.1	2.1 ± 0.1	Conforms to the description (Fig. 6)
TT2	11.3 ± 0.1	8.1 ± 0.1	1.9 ± 0.1	
TT3	9.4 ± 0.1	7.4 ± 0.1	1.6 ± 0.0	
TT4	11.1 ± 0.1	5.4 ± 0.1	1.2 ± 0.1	
	Quantification RA content (%)	**Extractives (%)**	**Foreign matter**	
			Stems (%)	**Other (%)**
TT1	0.42 ± 0.01	5.8 ± 0.1	3.3 ± 0.2	0.6 ± 0.1
TT2	0.83 ± 0.03	7.7 ± 0.2	2.6 ± 0.1	0.3 ± 0.1
TT3	0.53 ± 0.00	10.2 ± 0.1	2.9 ± 0.1	0.6 ± 0.3
TT4	0.81 ± 0.02	11.6 ± 0.1	1.5 ± 0.1	0.4 ± 0.1

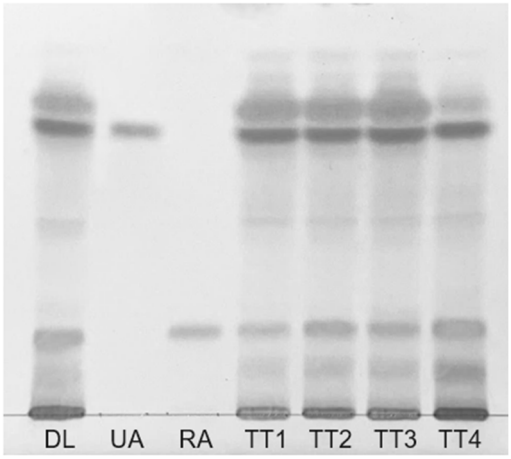

Fig. 6. TLC chromatograms for the identification of PS samples (TT1–TT4)
UA: ursolic acid; RA: rosmarinic acid; TT1-TT4: sample TT1-TT4; DL: Prunellae spica reference medicinal material

Descriptions of the medicinal herbs and powders from the four samples exhibited the morphological and microscopic characteristics described in Section 3.4. Sample TT1 showed the lowest ethanol-soluble extractive value (5.8% in 96% ethanol), which did not comply with the specified limit. In contrast, samples TT2, TT3, and TT4 met the extractive criterion, ranging from 7.7% to 11.6%. The RA content of all samples met both the in-house standard and the CP (2020) requirements, ranging from 0.42% to 0.83%. Overall, all four samples (TT1–TT4) satisfied the specifications of the developed in-house standard with respect to description, powder, water content, total ash, acid-insoluble ash, qualitative and quantitative identification, and foreign matter.

3.7. Discussion

The results for description, powder, water content, total ash, foreign matter, acid-insoluble ash, extractives, and identification by chemical reactions complied with the requirements specified in the *PS* monograph of the VP V. The VP V monograph does not include any marker compounds for either qualitative or quantitative analysis. In comparison, RA is used as a quality control marker for *PS* in the CP (2020), while

UA serves as a marker in the EP. In this study, both compounds were simultaneously examined to enhance the analytical scope of the in-house standard. However, due to the poor UV absorption of UA, only RA was quantified in accordance with the CP (2020) method, and UA was employed as a qualitative marker in the standard.

Quantitative analysis of both research and market samples revealed a wide variation in RA content (0.172% – 0.860%), which is consistent with previously reported studies. Several investigations worldwide have determined the RA content in PS mainly using HPLC. Liu et al. (2014) reported that RA content in 14 samples collected from different regions in China ranged from 0.0423 mg/g to 0.5687 mg/g [10]. RA has been identified as the major phenolic compound in PS, accounting for approximately 0.28% of the dried material, and its content increases slightly (to about 0.3%) when the influence of UV radiation is excluded [11]. Guo et al. (2012) further demonstrated that RA content varies with harvest time, reaching a maximum of 0.57% (on May 5) and a minimum of 0.17% (on June 25), suggesting that early May may represent the optimal harvest period for achieving superior quality [3]. Overall, the observed variability in the chemical quality of PS samples can be attributed mainly to differences in ecological conditions, geographical origins, and harvest periods.

4. Conclusions

An upgraded in-house quality standard for PS was established with the following specifications: description; powder; water content ≤ 12.0%; total ash ≤ 13.0%; acid-insoluble ash ≤ 3.0%; identification by chemical reactions and TLC (compared with the reference medicinal material and two reference substances RA and UA); quantification of rosmarinic acid by HPLC with a minimum required content of ≥ 0.20%, calculated with reference to the dried drug; extractives ≥ 7.0%; and foreign matter limited to stems ≤ 5.0% and other impurities ≤ 1.0%. The quality of several PS samples collected from the market was subsequently evaluated based on this in-house standard, demonstrating its applicability and reliability for routine quality assessment.

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