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Development of an HPTLC Method for Differentiating the Botanical Origins of Cuscutae Semen

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Abstract – Cuscutae Semen has a long-standing history as a traditional herbal medicine widely utilized in East Asian countries, including Korea, China, Hong Kong, and Taiwan. The Korean Herbal Pharmacopoeia (KHP) exclusively recognizes *Cuscuta chinensis* Lam. as the authentic source of Cuscutae Semen, whereas other countries also accept *Cuscuta australis* R.Br. This discrepancy has resulted in imports containing both species, which contravenes local regulations. Furthermore, a significant portion of domestically produced Cuscutae Semen is actually *Cuscuta japonica* Chois., regarded as an adulterant, which further complicates the market. This situation underscores the urgent need for clear differentiation of the botanical origins of Cuscutae Semen. In this study, a high-performance thin-layer chromatography (HPTLC) method was developed to simultaneously differentiate the three origins of Cuscutae Semen: *C. chinensis, C. australis*, and *C. japonica*. By utilizing a TLC scanner and TLC-MS interface, the chemical fingerprints of the samples were analyzed in detail. The results revealed that while *C. chinensis* and *C. australis* share similar profiles, they can be distinguished by the presence or absence of astragalin and kaempferol. In contrast, *C. japonica* showed distinct differences, characterized by the presence of chlorogenic acid derivatives. This method demonstrated the ability to rapidly and accurately differentiate between Cuscutae Semen origins, making it a cost-effective and reliable tool for ensuring quality control. It is expected to contribute to improving the standardization and reliability of herbal medicine quality management in the domestic market.

Keywords – High-performance thin-layer chromatography (HPTLC), Cuscutae semen, *Cuscuta chinensis* Lam., *Cuscuta australis* R.Br.,*Cuscuta japonica* Chois., TLC-MS

Introduction

Cuscutae Semen has a long history as an important herbal medicine, known for its diverse therapeutic effects, including strengthening kidney and liver functions, improving reproductive health, and protecting vision.[1] For example, it contains a high content of flavonoids and phenolic acids, such as rutin and astragalin, which are known for their potent antioxidant properties, protecting cells, and providing anti-inflammatory and anticancer effects.[2] In particular, the phenolic acids and flavonoids in Cuscutae Semen play a crucial role in the prevention of cardiovascular diseases and in exerting anti-inflammatory effects. For instance, rutin strengthens capillaries and helps lower blood pressure,[3] while hyperoside has strong antioxidant, anti-inflammatory, and anti-cancer effects while supporting key organs like the brain, cardiovascular system, kidneys, and liver, though prolonged use at high doses may cause renal toxicity.[4] Additionally, it contains polysaccharides and lignans that demonstrate immune-modulating, further supporting the therapeutic claims made by traditional Korean medicine.[5] The combined action of these bioactive compounds has established Cuscutae Semen as a valuable medicinal resource for the prevention and treatment of various diseases.

According to the Korean Herbal Pharmacopoeia (KHP), only *Cuscuta chinensis* Lam. (CC) is officially recognized as the authentic source of Cuscutae Semen. However, in the pharmacopoeias of countries such as China, Hong Kong, and Taiwan, both *C. chinensis* (CC) and *C. australis* R.Br. (CA) are accepted as the botanical origins. As a result, imports of these products into the domestic market often contain a mixture of both species, which is not compliant

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with local regulations. Even more concerning, a significant portion of domestically produced Cuscutae Semen in the market is actually the seed of *C. japonica* Chois. (CJ), which belongs to the Cuscuta family, but is considered an adulterant, further exacerbating uncertainty and disorder in the herbal medicine market. This growing confusion highlights the need for clear differentiation between the botanical origins of Cuscutae Semen.

Although CC, CA, and CJ are not easily distinguishable based on external morphology, there are subtle differences among them. CC is relatively larger, with a light brown to yellowish-brown surface and a diameter of around 3 mm. CA has a yellowish-brown surface with fine dots and white, thread-like markings visible under a magnifier. Its seeds have a diameter of 1.4–1.6 mm, and 100 seeds weigh approximately 100 mg. On the other hand, CJ varies from light brown to brown, featuring a beak-like protrusion at one end, a slightly sunken hilum on the lower side, and a diameter of approximately 0.7–2 mm.[6,7]

CJ differs from Cuscutae Semen due to variations in its chemical constituents. While Cuscutae Semen is known for its broad-spectrum activities such as anti-inflammatory, antioxidant, and hepatoprotective effects, CJ contains compounds like methyl caffeate, which primarily contribute to its antifungal properties. Therefore, CJ is regarded as a counterfeit product when used in place of Cuscutae semen.[1]

Various research efforts have been undertaken to differentiate the origins of Cuscutae Semen, with chemical fingerprinting and chemometric methods emerging as vital tools. High-Performance Liquid Chromatography (HPLC) fingerprinting is particularly useful in comparing the chemical profiles of different sources, aiding in the identification of Cuscutae Semen and detecting impurities or adulterants.[8–11]

High Performance Thin-layer Chromatography (HPTLC) is a simple and rapid analytical technique used to separate and identify compounds in a mixture, based on their different affinities for a stationary phase and a mobile phase. It offers significant advantages, such as high separation efficiency, selectivity, and the ability to analyze multiple samples simultaneously with simplified sample preparation.[12] To the best of our knowledge, there are no reports of studies using HPTLC to distinguish the adulterant CJ from Cuscutae Semen. Therefore, this study aims to develop an HPTLC method capable of identifying Cuscutae Semen by analyzing chemical fingerprints. Moreover, by utilizing a TLC scanner and TLC- MS interface, we confirmed the key components of CC, CA, and CJ, offering a reliable and efficient approach for ensuring its authenticity. This research is expected to make a significant contribution to the quality control of Cuscutae Semen, with HPTLC anticipated to play an important role in both identifying the origins and evaluating the quality of various herbal medicines used in traditional Korean medicine. As such, this study is poised to become a valuable tool for these purposes.

Experimental

Materials and reagents – Twenty-seven samples labeled as Cuscutae Semen were purchased from the domestic herbal medicine market in Korea, along with a sample labeled as CC (sample No.9) was provided by the Korean Ministry of Food and Drug Safety (MFDS), and a sample identified as CC (sample No.1) was obtained from the National Institute of Biological Resources (NIBR), making a total of 29 samples for the study. Details about the samples are presented in Table 1, with morphological images available in the supplementary data (Fig. S1). Hyperoside (quercetin 3-galactoside, CAS 482-36-0, purity 98.0%), astragalin (kaempferol 3-*O*-glucoside, CAS 480-10-4, purity 98.2%), isochlorogenic acid A (3,5-dicaffeoylquinic acid, CAS 2450-53-5, purity 98.6%), and chlorogenic acid (3-*O*-caffeoylquinic acid, purity 98.0%) were purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, China). Kaempferol (CAS 520-18-3, purity 99.7%) was obtained from Sigma-Aldrich (Steinheim, Germany).

For HPTLC analysis, ethyl acetate was supplied by Duksan Pure Chemicals Co., Ltd. (Ilsan, South Korea), and formic acid (99.0%) was provided by Daejung Chemicals & Metals Co., Ltd. (Siheung, South Korea). Also, for preparing the derivatizing reagent, methanol and acetic acid (99.0%) were obtained from Duksan Pure Chemicals Co., Ltd. (Ilsan, South Korea), while sulfuric acid (98.0%) and p-anisaldehyde were sourced from Sigma- Aldrich (Darmstadt, Germany). For the system suitability test (SST), the Universal HPTLC mix (UHM),[13,14] provided by CAMAG (Muttenz, Switzerland), which included paracetamol, thymidine, guanosine, sulisobenzone, phthalimide, 9-hydroxyfluorene, thioxanthen-9-one, and 2-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol, was utilized. Silica gel 60 F_{254} HPTLC plates (20 × 10 cm) were supplied by Merck (Darmstadt, Germany).

Instruments – The HPTLC analysis was performed using CAMAG (Muttenz, Switzerland) equipment, operated through visionCATS 3.2 software. The system was comprised of a Linomat 5 applicator equipped with a 100 μL syringe (CAMAG, Bonaduz, Switzerland) for sample application, TLC visualizer 3 for imaging chemical profiles, TLC scanner 4 for the qualitative analysis of standard compounds, and

Table 1. The detail information of the Cuscutae Semen samples

No.	Sample	Collected Year	Origin
01	*C. chinensis* (provided by NIBR) (Voucher No. NIBRGR0000431983)	2024	Korea (Yeongcheon-si)
02	Cuscutae Semen	2024	China
03	Cuscutae Semen	2024	China
04	Cuscutae Semen	2024	China
05	Cuscutae Semen	2024	China
06	Cuscutae Semen	2024	China
07	Cuscutae Semen	2024	China
08	*C. chinensis* (provided by MFDS)	2024	China
09	Cuscutae Semen	2024	China
10	Cuscutae Semen	2024	Korea (Chungju-si)
11	Cuscutae Semen	2024	Korea
12	Cuscutae Semen	2024	Korea
13	Cuscutae Semen	2024	Korea
14	Cuscutae Semen	2024	Korea (Wonju-si)
15	Cuscutae Semen	2024	Korea (Goesan-gun)
16	Cuscutae Semen	2024	Korea
17	Cuscutae Semen	2024	Korea
18	Cuscutae Semen	2024	Korea
19	Cuscutae Semen	2024	Korea
20	Cuscutae Semen	2024	Korea
21	Cuscutae Semen	2024	Korea
22	Cuscutae Semen	2024	Korea
23	Cuscutae Semen	2024	Korea
24	Cuscutae Semen	2024	Korea
25	Cuscutae Semen	2024	Korea
26	Cuscutae Semen	2024	Korea
27	Cuscutae Semen	2024	Korea
28	Cuscutae Semen	2024	Korea
29	Cuscutae Semen	2024	Korea

an automatic developing chamber (ADC) 2 with humidity control for chromatogram development. The chromatographic separation was conducted on HPTLC Silica gel 60 F_{254} plates. For mass spectrometry, CAMAG TLC-MS-interface with a binary pump (Hitachi, Ltd. Tokyo, Japan) was utilized to extract bands that either differentiated CC and CA from CJ or were common among them and subsequently inject into a mass spectrometer. The MS study was conducted using a JMS-T100TD (AccuToF-TLC) mass spectrometer (JEOL Ltd., Tokyo, Japan) equipped with Electrospray Ionization (ESI) mode.

Sample and standard solution preparation – The samples were uniformly ground and sieved using an 850 μm mesh to obtain an even particle size. We extracted each 1.0 g of powdered sample with 10 mL of methanol using ultrasonic extraction (700 W, Hwashin Tech., Daegu, South Korea) for 30 minutes. The resulting extract was filtered through filter paper (110 mm pore size, Hyundai Micro, Seoul, South Korea) and concentrated using a rotary evaporator (EYELA, Tokyo, Japan) with reduced pressure. The residue was reconstituted in 1 mL of methanol. For the standard solutions, each of hyperoside, astragalin, kaempferol, chlorogenic acid, and isochlorogenic acid A were prepared at a concentration of 500 μg/mL in methanol.

All sample and standard solutions were filtered through a syringe filter (Whatman, Maidstone, UK) featuring a 0.45 μm pore size.

Conditions for HPTLC analysis – Each band was applied as 2 μL, resulting in 8 mm bands. The track distance was set to 11.4 mm, and the first track was positioned 20 mm from the left edge. Additionally, the bands were placed 8 mm above the lower edge of the plate. The plate was developed up to 70 mm with a solvent system consisting of ethyl acetate, formic acid, and distilled water (15:1:1, v/v/v). Before development, the CAMAG glass twin trough chamber (20 × 10 cm, Muttenz, Switzerland) was saturated with a filter paper for 20 minutes. The development was performed under controlled conditions at 20 ± 5°C and 33 ± 5% relative humidity, maintained with a saturated $MgCl_2$ solution.

After development, the plate was derivatized using the CAMAG derivatizer. The plate was sprayed with the derivatizing reagent using a Blue nozzle at level 4 intensity and left to dry. The derivatizing reagent was prepared by slowly and carefully adding 10 mL of acetic acid followed by 5 mL of sulfuric acid sequentially to 85 mL of cold methanol, allowing the mixture to return to room temperature, then adding 0.5 mL of p-anisaldehyde. Once dried, the plate was heated using the CAMAG TLC plate heater III at 100°C for 3 minutes. The plate was immediately observed under 366 nm using a TLC visualizer and the UV spectra of hyperoside, astragalin, kaempferol, chlorogenic acid, and isochlorogenic acid A were obtained between 190 and 450 nm using a deuterium-tungsten lamp operating at a scanning speed of 20 mm/s with a slit dimension of 5 × 0.2 mm.

Conditions for TLC-MS analysis – The TLC-MS interface was used to extract target bands from the plate with 80% methanol as the solvent, delivered at a flow rate of 0.5 L/min. MS analysis was performed in negative ion mode under the following parameters: detector voltage at 2000 V, peak voltage at 1500 V, orifice 1 voltage set to −80 V at 80°C, orifice 2 voltage set to −10 V, and ring lens voltage set to −15 V. The desolvation chamber was maintained at 250°C, with nitrogen gas flow rates of 1.0 L/min for nebulization and 3.0 L/min for desolvation. The acquired m/z range was set between 50 and 1500, and prior to accurate mass measurements, the mass scale was calibrated using JEOL's YOKUDELNA calibration kit.

Results and Discussion

In this study, we successfully developed an HPTLC method that accurately differentiates the three origins of Cuscutae Semen (*C. chinensis*, *C. australis*, and *C. japonica*), commonly distributed in the domestic market. However, distinguishing between the two herbal origins, CC and CA, presented significant challenges since their HPTLC chemical fingerprints were very similar, whereas the fingerprint of CJ was markedly different (Fig. 1). In the group of CC and CA, hyperoside was detected at R_f 0.25 as a blue fluorescent band in both samples. Astragalin, and kaempferol were observed only in CA at R_f 0.35 and 0.82, respectively. Isochlorogenic acid A appeared in both CC and CJ at R_f 0.63, while chlorogenic acid, found at R_f 0.22, was detected only in CJ samples. Each band was identified by comparing the R_f values from the fingerprints and spectra obtained from the TLC scanner with those of the standard compounds (Fig. 2).

A System Suitability Test (SST) was conducted using a Universal HPTLC mix (UHM) to evaluate the consistency of the HPTLC analysis. The UHM provided 5 clear reference bands with R_f values of 0.84, 0.69, 0.29, 0.13 and 0.02, with an R_f deviation of less than 0.11, observed under UV 254 nm. The results of the SST are detailed in the Supplementary Data (Table S1 and Fig. S2).

To further clarify the compounds present in each group, a TLC-MS interface was employed. We confirmed the MS values of each band of hyperoside, astragalin, kaempferol, chlorogenic acid, and isochlorogenic acid A. Hyperoside, astragalin, and kaempferol were detected in negative mode as $[M-H]^-$ ions, with *m/z* values of 463.08828 for

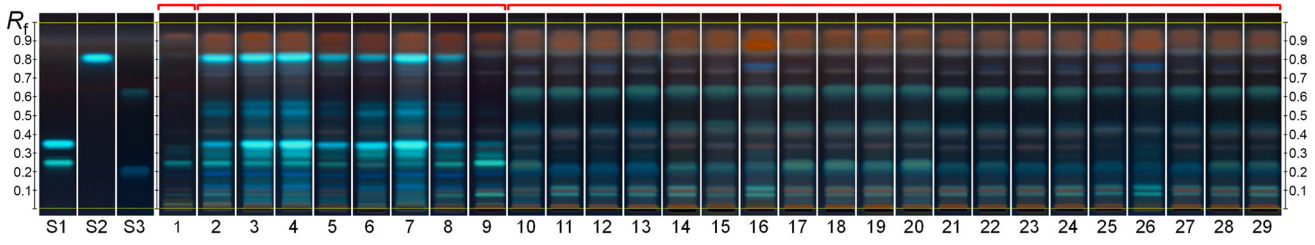

Fig. 1. HPTLC chromatogram of the CC(1), CA(2-9) and CJ (10-29) visualized using the method under UV light at 366 nm; S1: hyperoside(lower) and astragalin(upper) (500 μg/mL), S2: kaempferol (500 μg/mL), S3: chlorogenic acid(lower) and isochlorogenic acid A(upper) (500 μg/mL).

hyperoside, 447.09109 for astragalin, and 285.03878 for kaempferol, which correspond to the group of CC and CA. Chlorogenic acid and isochlorogenic acid A were detected in the same mode, as $[M+Na-2H]^-$ ions, with *m/z*

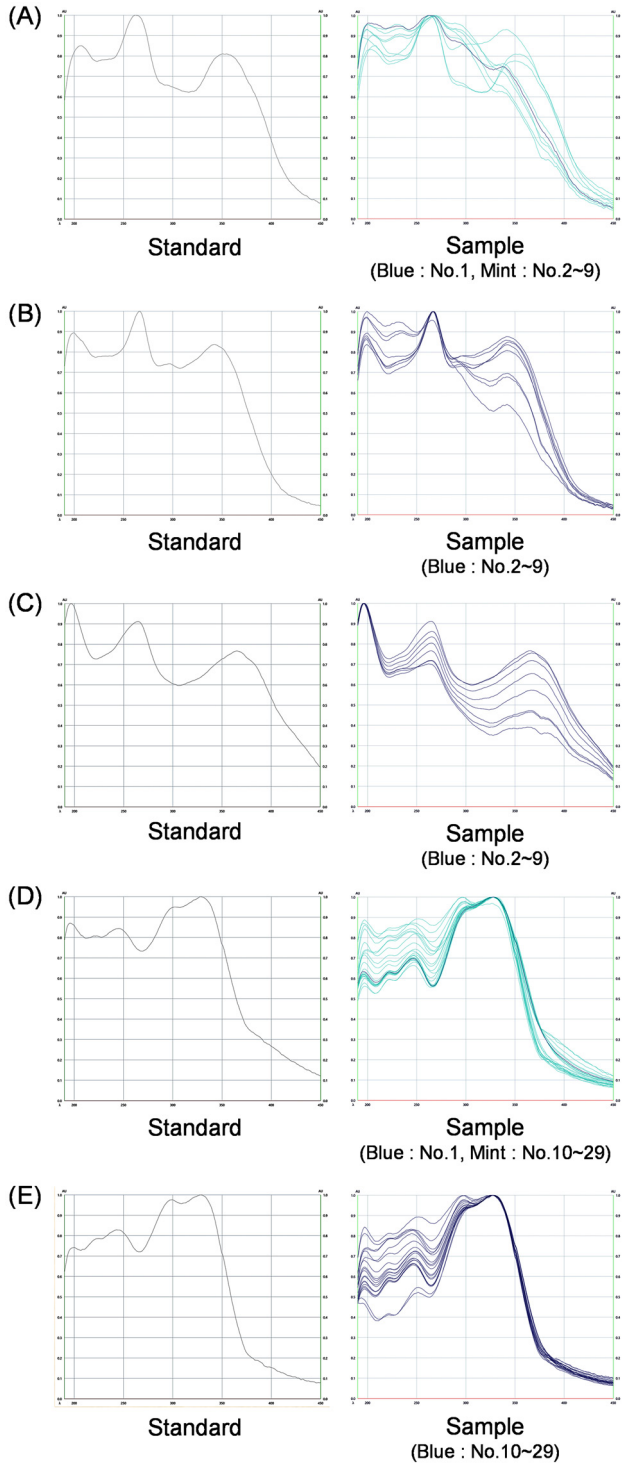

Fig. 2. UV spectra of (A) hyperoside, (B) astragalin, (C) kaempferol, (D) isochlorogenic acid A, (e) chlorogenic acid standard bands and the corresponding bands in the sample solution.

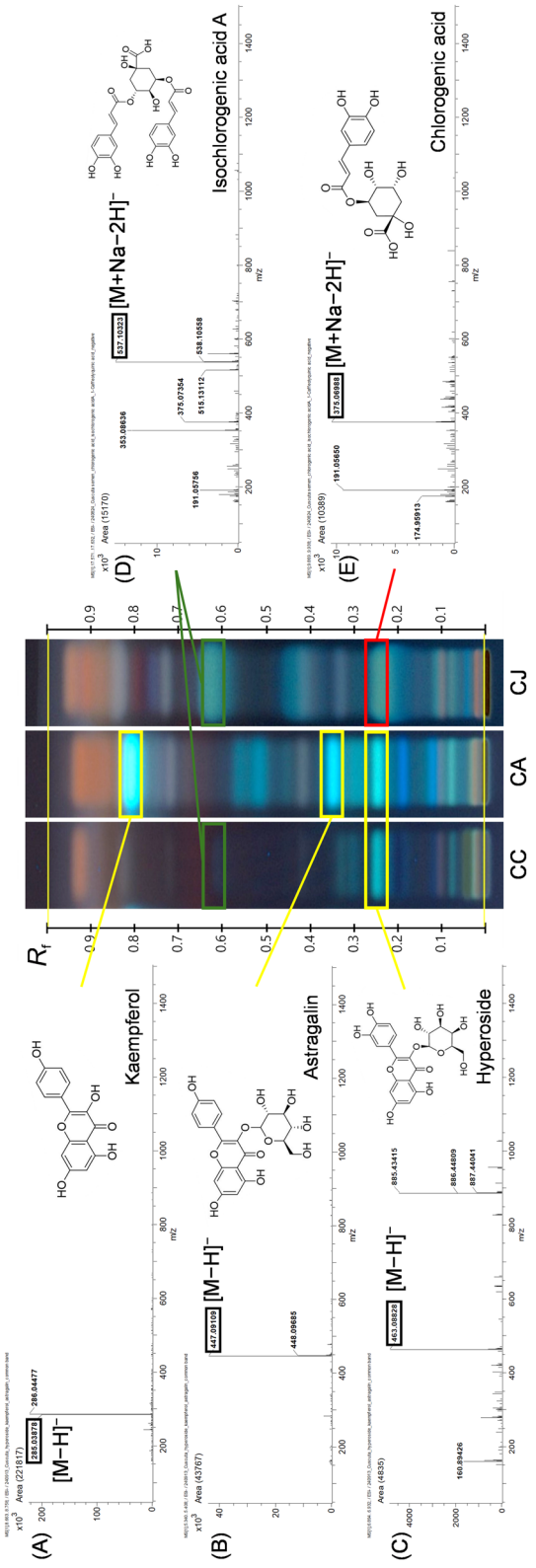

Fig. 3. TLC-MS spectra of the five standard bands in the chemical fingerprints of CC(sample No.1), CA(sample No.2) and CJ(sample No.10); kaempferol at R_f 0.82, astragalin at R_f 0.35, hyperoside at R_f 0.25, isochlorogenic acid A at R_f 0.63, and chlorogenic acid at R_f 0.22.

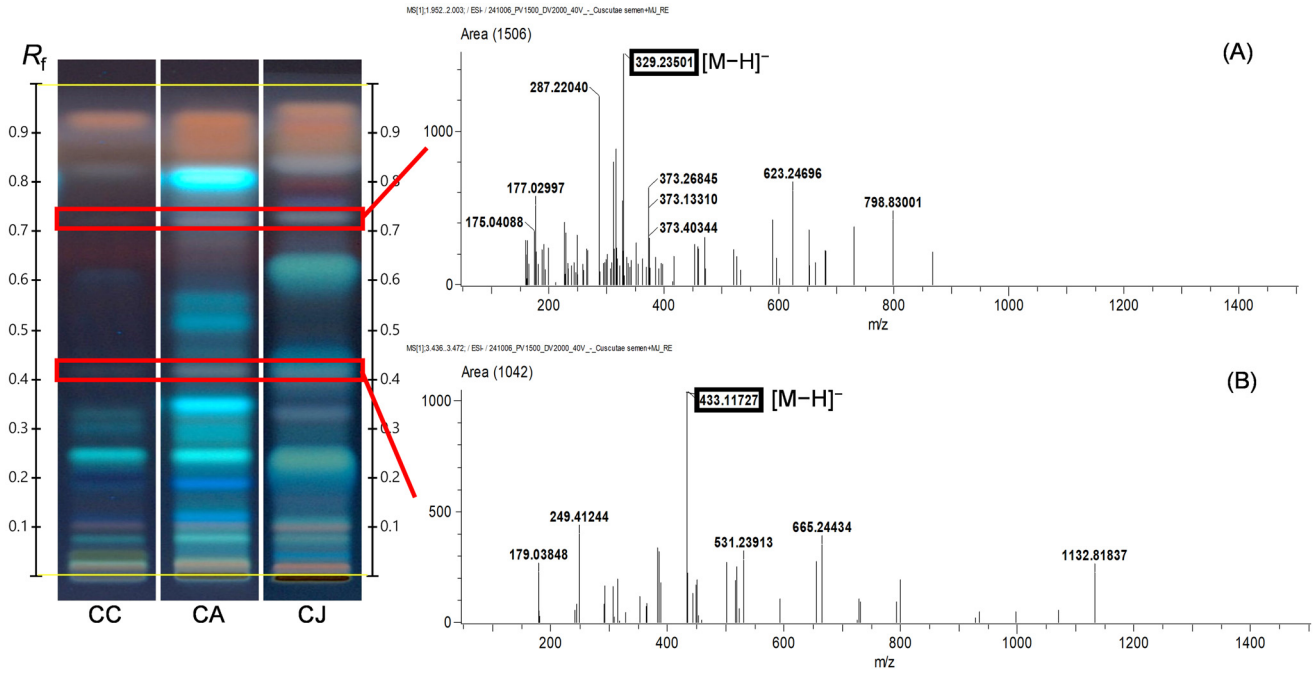

Fig. 4. TLC-MS spectra of the common bands in the chemical fingerprints of both CC (sample No.1), CA (sample No.2) and CJ (sample No.10); a band at R_f 0.72, and a band at R_f 0.42.

values of 375.06988 for chlorogenic acid, 537.10323 for isochlorogenic acid A, all of which matched the expected values (Fig. 3). Additionally, the mass spectra of bands common to all samples, observed at R_f 0.42, and R_f 0.72 were presented. For R_f 0.72, we obtained an *m/z* of 329.23501 in the $[M-H]^-$ form, and for R_f 0.42, we measured an *m/z* of 433.11727 in the same form (Fig. 4). Although these compounds have not yet been identified, further research to identify these compounds could deepen our understanding of the metabolomic profiles of Cuscuta family.

In conclusion, this study demonstrated that HPTLC analysis is an effective tool for economically and efficiently distinguishing the origins of Cuscutae Semen. The developed method offers high separation efficiency, simplified sample preparation, and the ability to process multiple samples simultaneously, making it a practical solution for routine quality control. This advancement is expected to contribute significantly to the standardization and quality assurance of Cuscutae Semen products in both domestic and international markets.

The analysis was conducted using sample No.1, provided by NIBR, as a reference for CC, as it was confirmed to be CC through DNA analysis.[1] While CC and CA shared similar chromatograms, samples containing kaempferol, based on previous studies,[1] were identified as CA. Among the Cuscutae Semen samples purchased from the domestic market, 20 samples, which originated from Korea, were identified as the adulterant CJ, suggesting that the majority of Cuscutae Semen circulating domestically is the adulterant species. These findings highlight that only one of the 29 samples analyzed matched the origin officially recognized in Korea, CC, underscoring the widespread distribution of unapproved origins. Therefore, systematic management and stricter quality control measures are essential to prevent adulteration and ensure the authenticity of Cuscutae Semen in the domestic market.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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