การเปรียบเทียบวิธีเพื่อระบุชนิดพืชที่ปลอมปนในผลิตภัณฑ์สมุนไพรฟ้าทะลายโจร ระหว่างวิธีการตรวจลักษณะทางจุลทรรศน์ วิธีโครมาโทกราฟีชนิดผิวบางสมรรถนะสูง (HPTLC) และวิธีการแตกตัวของไอออนด้วยเลเซอร์แบบเมทริกซ์

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บทคัดย่อ การแพร่ระบาดของโรคโควิด 19 ระหว่างปี พ.ศ. 2562–2564 ทำให้ความต้องการใช้สมุนไพรฟ้าทะลายโจร (*Andrographis paniculata* (Burm.f.) Nees; AP) เพิ่มสูงขึ้นอย่างมาก นำไปสู่การปลอมปนของพืชอื่นในผลิตภัณฑ์ฟ้าทะลายโจร การศึกษานี้มีวัตถุประสงค์เพื่อตรวจสอบความถูกต้องของผลิตภัณฑ์ฟ้าทะลายโจร โดยซื้อตัวอย่างจากร้านขายยาแผนโบราณและร้านสมุนไพรในท้องถิ่น จำนวน 13 ตัวอย่าง และเป็นตัวอย่างจากกองบังคับการปราบปรามการกระทำความผิดเกี่ยวกับการคุ้มครองผู้บริโภค (บก.ปคบ.) จำนวน 1 ตัวอย่าง โดยใช้การตรวจสอบหลายเทคนิค ประกอบด้วย การตรวจลักษณะทางจุลทรรศน์ การวิเคราะห์ด้วยโครมาโทกราฟีชนิดผิวบางสมรรถนะสูง (HPTLC) และการตรวจระดับชีวโมเลกุลด้วยเทคนิค MassARRAY-MALDI-TOF การตรวจทางจุลทรรศน์ยืนยันว่ามีหลายตัวอย่างที่ไม่ใช่ฟ้าทะลายโจร การตรวจด้วยวิธี HPTLC ต้องพบสารแอนโดรกราโฟไลด์ซึ่งเป็นสารชี้บ่งในสมุนไพรชนิดนี้ จากการศึกษาพบว่ามีจำนวน 11 ตัวอย่างที่มีลักษณะโครมาโทกราฟีสอดคล้องกับฟ้าทะลายโจรแท้ มี 3 ตัวอย่าง ไม่พบสารดังกล่าว และการตรวจสอบด้วยเทคนิค MassARRAY พบการปลอมปนใน 11 ตัวอย่าง โดยระบุได้ว่าเป็นฟ้าทะลายโจรแท้ 3 ตัวอย่าง เป็นสะเดา (*Azadirachta indica* A. Juss) 2 ตัวอย่าง เป็นส่วนผสมของฟ้าทะลายโจร สะเดา และบอระเพ็ด (*Tinospora crispa* (L.) Miers ex Hook.f. & Thomson) 8 ตัวอย่าง และตัวอย่างจาก บก.ปคบ. พบว่าเป็นบอระเพ็ด การศึกษานี้ชี้ให้เห็นว่าแม้วิธี HPTLC จะเหมาะสำหรับการคัดกรองเบื้องต้น แต่เทคนิค MassARRAY มีความไวและความจำเพาะสูงกว่าสำหรับการยืนยันความแท้ของสมุนไพร การบูรณาการตรวจสอบโดยใช้สามวิธีร่วมกันจะช่วยเพิ่มความน่าเชื่อถือในการพิสูจน์ชนิดสมุนไพรและคุ้มครองผู้บริโภคจากการปนปลอมของผลิตภัณฑ์ฟ้าทะลายโจร

คำสำคัญ: ฟ้าทะลายโจร, การปลอมปนในผลิตภัณฑ์สมุนไพร, การตรวจลักษณะทางจุลทรรศน์, HPTLC, MassARRAY

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Introduction

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019 triggered an unprecedented global health crisis, culminating in the COVID-19 pandemic.[1] This encapsulated, single-stranded RNA virus spreads rapidly and primarily targets the respiratory system, leading to a spectrum of clinical manifestations ranging from mild symptoms to severe pneumonia and acute respiratory distress syndrome.[2,3] In response to the urgent need for effective therapeutics, traditional medicinal plants have garnered renewed attention due to their rich source of bioactive compounds, such as flavonoids, alkaloids, terpenoids, and polysaccharides,[3,4] which exhibit antiviral, anti-inflammatory, and immunomodulatory properties. Herbal medicines are well-known for their antiviral, anti-inflammatory, and immunomodulatory capabilities. They are presently the focus of research targeted at reducing SARS-CoV-2 infection.

Among these, *Andrographis paniculata* (Burm.f.) Nees (AP) has long been utilized in traditional medicine across Asian countries[5] and has recently gained prominence as a potential candidate for COVID-19 treatment. Studies suggest that its active constituents may disrupt the viral life cycle by interacting with viral proteins and impeding viral entry into host cells.[6,7] Furthermore, AP showed immunomodulatory effects that enhance host defenses and regulate inflammatory responses, offering a dual mechanism to mitigate both the symptoms and complications of COVID-19.[6-8]

To reflect the therapeutic landscape between 2019 and 2023, before the evolution of current treatment guidelines, the following discussion highlights the exploratory role of AP and related botanicals in antiviral research. At that time, growing interest in the pharmacological properties of AP and its principal bioactive compound, andrographolide, underscored the potential of these natural agents to complement antiviral therapies. While preliminary findings suggested promising antiviral activity, particularly against respiratory viruses, the clinical efficacy of AP remained to be substantiated through rigorous trials. Enhancing the bioavailability of andrographolide was recognized as a critical step toward optimizing its therapeutic value. The investigation of phytochemicals such as AP continued to represent a viable strategy for novel antiviral drug development. Notably, the Ministry of Public Health, Thailand, endorsed the use of AP for the treatment of mild COVID-19 cases during this period, further emphasizing its perceived utility in pandemic response efforts.[9] However, the surge in demand coupled with limited AP raw material availability has led to widespread adulteration and substitution with other bitter botanicals, including *Momordica charantia, Tinospora crispa,* and *Azadirachta indica.* Conventional quality control methods, such as morphological, chemical, and genetic analyses, each present limitations when applied to processed herbal materials, raising serious concerns regarding product authenticity, efficacy, and safety.[10-12]

Microscopic examination allows direct observation of diagnostic features; nevertheless, it is less useful on processed materials, such as powders or extracts, where important structures are obscured.[13] Chemical profiling techniques, such as high-performance thin-layer chromatography (HPTLC), provide consistent

fingerprints of marker compounds;[14,15] however, their analysis may be limited by the availability of reference standards and the similarity among closely related species.[16-18] Genetic profiling has emerged as a powerful tool for plant authentication, particularly when morphological and chemical methods are inconclusive due to post-harvest changes or species resemblance.[19] Recent advances, such as the technique integrating Multiplex-PCR with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF Mass Spectrometry) (MassARRAY),[20] have significantly enhanced botanical authentication by enabling rapid and sensitive detection of single nucleotide polymorphisms (SNPs). The MassARRAY iPLEX assay is a sophisticated, high-throughput platform used for analyzing SNPs, making it ideal for accurately authenticating botanicals. The technique leverages the precision of mass spectrometry to differentiate DNA based on minute changes in molecular weight, thereby confirming species identity.[21,22] The process begins with specific PCR amplification, where the initial step isolates and amplifies the DNA region containing the target SNP using specific primers. Following amplification, the DNA fragments proceed to the single-base extension (iPLEX), where a single Extension Primer is designed to anneal immediately upstream of the SNP site. Using a modified DNA polymerase, a single, mass-modified nucleotide (A, G, C, or T) is incorporated into the primer, complementing the template base at the SNP position. Because each of the four bases has a unique mass, this incorporation results in a precise, predictable change in the overall mass of the primer product. Finally, the extended products are analyzed via Mass Spectrometry Detection (MALDI-TOF MS), where the extended products are dispensed onto a chip and analyzed by a MALDI-TOF Mass Spectrometer. The instrument precisely measures the molecular mass of the extended primer. The detected mass signal is directly translated into a "Base Call" (EXT Call), revealing the specific nucleotide present at that SNP location. This ability to generate a clear mass signature for each plant species, even from highly processed or mixed samples, provides the definitive molecular evidence necessary to detect and identify adulteration that morphological or chemical methods may fail to resolve.

However, their application in herbal authentication is yet inadequately investigated, especially in intricate compositions. When used alone, each of these approaches has its problems. For example, chemical adulteration is difficult to detect with a microscope, structural similarity is challenging to observe with HPTLC, and MassARRAY technology requires extensive validation.[21-24] To ensure accurate identification of herbal ingredients in complex formulations, various analytical techniques have been employed, each with distinct advantages and limitations. Microscopic characteristics offer rapid and low-cost screening that aligns with pharmacopoeial standards but are often unsuitable for processed products such as powders or capsules, where structural markers may be lost. HPTLC provides reproducible phytochemical fingerprints and is widely accepted in regulatory quality control. Moreover, the specificity of analysis may be limited in polyherbal mixtures due to band overlap among related species. MassARRAY technology delivers high sensitivity and specificity, particularly in processed or complex samples, though it requires advanced instrumentation and a comprehensive spectral library. An

integrated approach, combining complementary methods, could overcome individual limitations, offering cross-validated evidence and enhanced reliability.

This study aimed to compare a newly developed MassARRAY-based single nucleotide polymorphism (SNP) genotyping approach with conventional methods, including microscopic identification and high-performance thin-layer chromatography (HPTLC) fingerprinting, for the authentication of *A. paniculata* raw materials and herbal products. The MassARRAY technique was introduced as a novel molecular tool designed to overcome the inherent limitations of morphological and chemical analyses. Through a systematic comparison of analytical performance, this study sought to establish an integrated and regulation-compliant framework that enhances accuracy, reproducibility, and reliability in herbal authentication. Ultimately, the comparative evaluation not only demonstrated the potential of the MassARRAY platform as a robust complementary method but also provided a reference model for improving the quality control of herbal medicines.

Materials and Methods

Chemicals and Materials

Andrographolide, purity 98.5% w/w (Chromadex, USA), toluene, AR (Merck, Germany), ethyl acetate, AR (Merck, Germany), dichloromethane, AR (Merck, Germany), formic acid, AR (Merck, Germany), methanol, AR and LC (Merck, Germany), chloroform, AR (Labscan, USA), sulfuric acid, 95–98.6%, AR (Merck, Germany), deionized water, ultrapure, type I, and HPTLC glass plates, silica gel 60 F_{254} (Merck, Germany), anisaldehyde (Sigma-Aldrich, USA), DNA clean-up kit (BioFact Genomic DNA Prep Kit, Republic of Korea), cetyltrimethylammonium bromide (CTAB), molecular (Himedia, India), and β-mercaptoethanol (Sigma-Aldrich, USA).

Equipment

TLC densitometer (CAMAG, Switzerland), Analytical balance (Mettler Toledo, Switzerland). Water bath (Memmert, Germany), Nanophotometer N80 (Prima Scientific, USA). Microscope (Olympus BX43 light, Japan), Ultra-Performance Liquid Chromatography (Waters, USA), MassARRAY-Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry analyzer (Agena Bioscience, USA), Reverse-phase column, Acquity UPLC BEH C18 2.1 mm ID × 50 mm, 1.7 μm (Waters, USA), and UPLC (Waters, USA).

Authentic Plant Specimens and Samples

Authentic plant specimens were collected and identified [Herbarium at the Medicinal Plant Research Institute, Department of Medical Sciences, Thailand (DMSc)]; *Andrographis paniculata* (Burm.f.) Nees, (AP) (DMSc number 5392) leaves, *Azadirachta indica* A. Juss., (AI) (DMSc number 5442) leaves, and *Tinospora crispa* (L.) Miers ex Hook.f. & Thomson (TC) (DMSc number 5356) leaves. One unknown sample was received from the Consumer Protection Police Division (CPPD), and 13 unknown samples in the form of capsules were purchased from traditional drugstores and herbal markets. The dried authentic samples were powdered, passed through the 150 micron sieve, and stored in airtight containers. All 14 unknown samples were passed through the 150 micron sieve and stored in airtight containers. The details and sources of the sample are shown in Table 1.

Table 1 Authentic specimens and unknown sample details, and sources of samples

No.	Sample name	Detail	Type	Source	Andrographolide content (% w/w)
1	AP	AP	Powder	DMSc	1.82±0.00
2	AI	AI	Powder	DMSc	n.d.
3	TC	TC	Powder	DMSc	n.d.
4	Unk 01	Herbal market	Capsule	Lop Buri	n.d.
5	Unk 02	Drugstore	Capsule	Saraburi	0.97±0.02
6	Unk 03	Drugstore	Capsule	Chumphon	1.06±0.00
7	Unk 04	Drugstore	Capsule	Udon Thani	1.33±0.00
8	Unk 05	Herbal market	Capsule	Suphan Buri	0.35±0.02
9	Unk 06	Herbal market	Capsule	Nonthaburi	0.41±0.02
10	Unk 07*	Suspected capsule	Capsule	CPPD	n.d.
11	Unk 08	Herbal market	Capsule	Suphan Buri	0.26±0.02
12	Unk 09	Herbal market	Capsule	Roi Et	n.d.
13	Unk 10	Drugstore	Capsule	Bangkok	0.85±0.01
14	Unk 11	Drugstore	Capsule	Bangkok	0.94±0.00
15	Unk 12	Drugstore	Capsule	Lampang	1.00±0.00
16	Unk 13	Drugstore	Capsule	Songkhla	1.20±0.00
17	Unk 14	Herbal market	Capsule	Nonthaburi	1.33±0.00

Note: *Suspected sample received from the Consumer Protection Police Division (CPPD), n.d. (not detected) indicates concentration of andrographolide below the LOD, 3.2 μg/mL

Microscopic Examination

The macroscopic and microscopic diagnostic characteristics of authentic *A. paniculata* (AP), as well as *A. indica* (AI), *T. crispa* (TC), and suspected capsules from CPPD and 13 unknown capsule samples, were analyzed. The samples, 2 mg each, were mounted with glycerin and examined under a microscope. Microscopic examination of the powdered samples was conducted at 40x magnification, and photographs were taken for documentation. Each slide was prepared in 10 replicates. Key diagnostic microscopic features of unidentified samples were assessed under these conditions. The Thai Herbal Pharmacopoeia (THP) monograph was utilized as a reference for the identification of AP. The physical characteristics of the sample powders, including color, smell, and taste, were also recorded.

High-Performance Thin-Layer Chromatography (HPTLC) Analysis

One hundred mg of the sample was extracted with 1.0 mL of methanol for 10 minutes in a water bath and then filtered through a 0.22 μm filter before use. Two μL of the filtrates was applied to the HPTLC plate as a 6 mm band using a TLC sampler. The band was positioned 8 mm apart from other bands and 8 mm above the bottom of the plate. HPTLC separation using solvent system I consisted of a mixture of dichloromethane, ethyl acetate, and methanol (4:3:0.4, v/v/v), while solvent system II consisted of toluene and ethyl acetate (3:7, v/v) was in an unsaturated 20 cm × 10 cm Twin Trough Chamber with a migration distance of 80 mm. After development, the plate was dried with cool air. The developed bands were detected

under 254 and 366 nm. Their hR_F values were determined, and the HPTLC chromatogram was captured using the TLC Visualizer Documentation System. The developed chromatogram was sprayed with anisaldehyde-sulfuric acid reagent and heated at 105°C for 5 minutes HPTLC profiles were analyzed under white light following derivatization with an anisaldehyde–sulfuric acid reagent.

Ultra-Performance Liquid Chromatography (UPLC) Analysis

Approximately 500 mg of the accurately weighed sample was refluxed with 50 mL of a dichloromethane–methanol (1:1, v/v) mixture for 30 minutes. The extract was filtered, and the filtrate was evaporated to dryness. The residue was reconstituted in methanol and adjusted to a final volume of 25 mL. A 5 mL aliquot of this solution was further diluted to 10 mL with water and filtered prior to LC injection. The quantification of andrographolide content was conducted using the UPLC system equipped with a binary pump, vacuum degasser, autosampler, and column oven. The analyte was detected by a 2996 photodiode array detector and analyzed using Empower 2 software (Waters, USA). A 2 µL sample was injected into a reverse-phase column, eluted with methanol (solvent A) and DI water (solvent B). Isocratic elution at a 50:50 ratio of solvents A and B was performed at a flow rate of 0.6 mL/min for 2.5 minutes and detected at 226 nm, while the column temperature was maintained at 35°C. The andrographolide content was quantified using the linear regression equation obtained from the calibration curve.

Single Nucleotide Polymorphism (SNP) Identification Using the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MassARRAY-MALDI-TOF)

Total DNA Extraction

Total genomic DNA was extracted from authentic herbal powder samples (AP, AI, and TC) and other unknown samples. For DNA extraction, 0.3–0.5 g samples were used, employing the modified CTAB method.[25-28] The samples were incubated with CTAB buffer and ß-mercaptoethanol at 55°C for 20 minutes. Chloroform:isoamyl alcohol (24:1) was added to separate the phases, and the mixture was centrifuged at 12,000 rpm for 3 minutes at 4°C. The aqueous phase was transferred to a new tube, and 500 µL of absolute ethanol and 3 M sodium acetate were added. The mixture was centrifuged at 12,000 rpm at 3 minutes at 4°C. Then, the supernatant was discarded, the pellet was washed with 75% ethanol, and the mixture was centrifuged at 12,000 rpm for 3 minutes at 4°C. The supernatant was then discarded. The pellet was air-dried at room temperature, resuspended in the elution buffer, and purified using a DNA clean-up kit (BioFact Genomic DNA Prep Kit, Republic of Korea). The quality and quantity of the DNA were analyzed using agarose gel electrophoresis and the Nano-photometer N80. The DNA samples were stored at −80°C for further experiments.

Verification of Authentic Plant Materials

The identity of the authentic plant materials [*A. paniculata* (AP), *A. indica* (AI), and *T. crispa* (TC)] was initially verified using

DNA barcoding prior to SNP analysis. The *rbcL* gene was selected as the genetic marker and was amplified using the universal primers rbcLa-F (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) and rbcLa-jf634R (5′-GAAACGGTCTCTCCAACGCAT-3′)[19,29] and subsequently sequenced. Sequence homology searches were performed by comparing the resulting sequences with existing reference sequences in the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST). Only samples showing a sequence homology of ≥ 99% with the known species sequences were designated as authenticated references for the subsequent assay development.

Development of MassARRAY-based SNP Assay

Specific primers for SNP detection were then designed to distinguish AP from the potential adulterants AI and TC using the MassARRAY-based assay. The design utilized the online MassARRAY Assay Design software (Agena Bioscience, https://ads.agenabio.com/AssayDesignerSuite.html), based on the *rbcL* gene sequences generated from the verified authentic samples (Figure 1), which were aligned against reference sequences from the NCBI GenBank database. The resulting primer sequences and their corresponding target SNP positions are detailed in Table 2. The designed primers were tested against the authentic AP, AI, and TC DNA samples in a crucial verification step to establish the unique and definitive mass signatures (EXT Calls) for each species at the selected SNP positions, as shown in Figure 1.

Subsequently, the authentic and unknown samples were assessed using the MALDI-TOF MS method according to the manufacturer's protocol. Step 1: PCR reaction preparation. The reaction consisted of 50 ng DNA, PCR buffer, magnesium chloride, dNTPs, Taq polymerase, and primers (rbcLa-F and rbcLajf634-R). Step 2: Shrimp Alkaline Phosphatase (SAP) reaction, which involves dephosphorylating unincorporated dNTPs. The reaction included the PCR reaction, SAP buffer, and SAP enzyme. Step 3: Extension reaction: the SAP reaction was further processed with iPlex Pro buffer, iPlex Pro terminator mix, iPlex Pro Enzyme, and extension primers (Table 2). After PCR reaction, the PCR products were transferred to a 96-well plate, and 29 μL of Type I ultrapure water was added before proceeding to the analysis. Using the MassARRAY analyzer, four PCR product samples were loaded onto the SpectroCHIP (iPLEX Pro SampleID Panel Set, Agena Bioscience, San Diego, USA) after resin desalination (iPLEX Pro SampleID Panel Set, Agena Bioscience, San Diego, USA). Then, the obtained mass spectrometry data for the SNP targets were analyzed using the Typer 4 Analyzer software (Agena Bioscience, San Diego, USA).

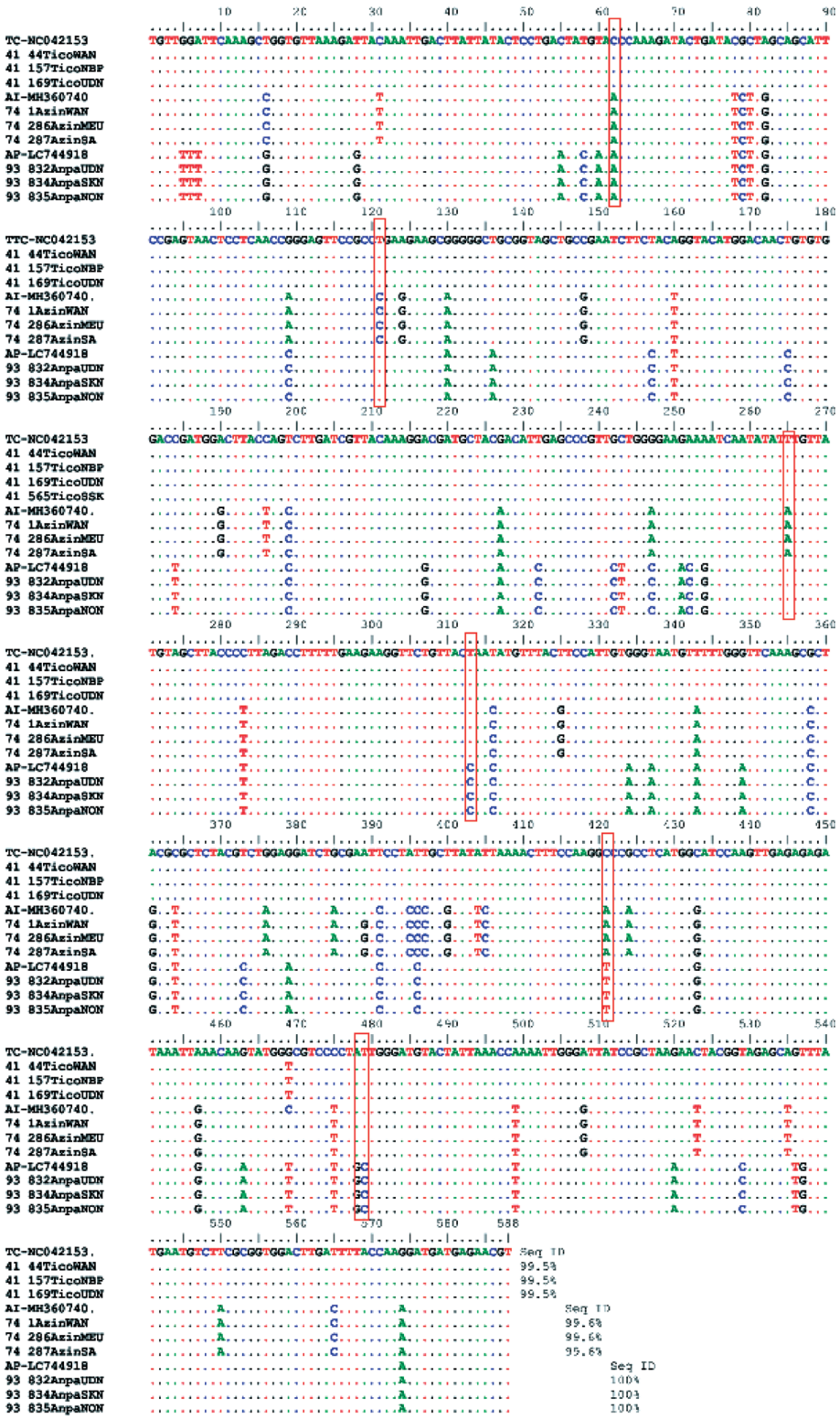


Figure 1 Multiple sequence alignment of the *rbcL* gene region for species verification and SNP marker selection. The alignment displays *rbcL* gene sequences obtained from the authentic plant samples generated in this study: *A. paniculata* (AP; 93 832AnpaUDN, 93 834AnpaSKN, 93 835AnpaNON), *A. indica* (AI; 74 1AzinWAN, 74 286AzinMEU, 74 287AzinSA), and *T. crispa* (TC; 41 44TicoWAN, 41 157TicoNBP, 41 169TicoUDN). These sequences are juxtaposed with corresponding reference sequences from the NCBI

GenBank database (NC042153, LC744918, and MH360740). The Sequence Identity (Seq ID) values indicate the percentage of nucleotide similarity compared to the closest reference sequence, thereby confirming the identity and purity of the authentic materials. The seven positions (62 = TC1, 121 = AI2, 265 = AI7, 313 = AP14, 421 = AAT4, and 478–479 = AP21), which are demarcated by red square boxes, represent the functional SNP markers selected for the final MassARRAY–MALDI–TOF assay due to their clear species–specific polymorphisms.

Table 2 Sequence, molecular mass, extension variant call (EXT Call), and read of extension primers for SNP detection in a mixture powder of three herbal plants

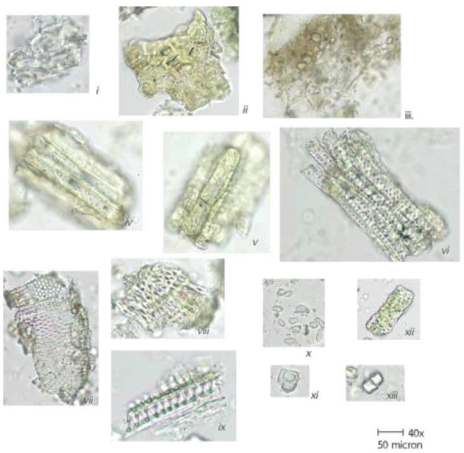
Primer ID	(5′⇋3′) Sequence	Molecular mass	EXT Call	Read
AAT4	F-gATTAAAACTTTCCAAGG	5507.6	No call	UEP
	F-gATTAAAACTTTCCAAGGC	5754.8	C	TC
	F-gATTAAAACTTTCCAAGGA	5778.8	A	AI
	F-gATTAAAACTTTCCAAGGT	5834.7	T	AP
AI2	F-ccccACCAGGAGTTCCGCC	5695.7	No call	UEP
	F-ccccACCAGGAGTTCCGCCC	5942.9	C	AI
	F-ccccACCAGGAGTTCCGCCT	6022.8	T	AP, TC
AI7	R-gAGGGTAAGCTACATAACA	5870.9	No call	UEP
	R-gAGGGTAAGCTACATAACAA	6142.1	T	AP, TC
	R-gAGGGTAAGCTACATAACAT	6197.9	A	AI
AP14	F-AGAAGGTTCTGTTAC	4608.0	No call	UEP
	F-AGAAGGTTCTGTTACC	4855.2	C	AP
	F-AGAAGGTTCTGTTACT	4935.1	T	AI, TC
AP21	R-TTTAATAGTACATCCCA	5129.4	No call	UEP
	R-TTTAATAGTACATCCCAA	5400.6	AT	AI, TC
	R-TTTAATAGTACATCCCAG	5416.6	GC	AP
TC1	R-ATCAGTATCTTTGG	4269.8	No call	UEP
	R-ATCAGTATCTTTGGG	4557.0	C	TC
	R-ATCAGTATCTTTGGT	4596.9	A	AP, AI

Note: AP = *A. paniculata*, AI = *A. indica*, TC = *T. crispa*, UEP = un–extension primer

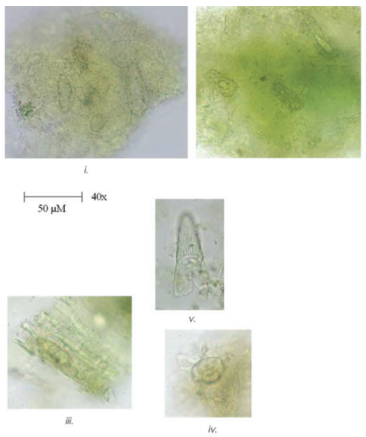
Results

Microscopic Features

The unknown sample from CPPD (A) displayed heterogeneous anatomical features suggestive of mixed or non-*Andrographis paniculata* (AP) plant tissues. Key structures observed included: upper epidermis (i); parenchyma cells (ii), some containing prismatic crystals, as well as prismatic crystals in the sectional view of lamina (iii), anomocytic stomata (iv), starch grains (iii); xylem parenchyma (iv); sclereids (v); lignified parenchyma (vi); bordered-pitted vessels (vii); reticulate vessels (viii); spiral vessels (ix); free starch grains (x); parenchyma cells containing prismatic crystals (xi); stone cells (xii); and prismatic crystals features (xiii) inconsistent with authentic AP (Figure 2A). In comparison, microphotographs of authentic *A. paniculata* (B) showed an upper epidermis with lithocysts containing cystoliths and multicellular glandular trichomes (i), cystoliths (ii), diacytic stomata (iii), multicellular glandular trichomes (iv), and parts of uniseriate multicellular trichomes (v) (Figure 2B). *Azadirachta indica* (C) exhibited an upper epidermis of polygonal cells (i), some containing brown substances (ii), associated with bulliform cells (iii), and a lower epidermis composed of polygonal cells with normocytic stomata, multicellular glandular trichomes (iv), bulliform cells (v), and rosette aggregate crystals (vi) (Figure 2C). *Tinospora crispa* (D) showed an upper epidermis (i) and parenchyma (ii) containing prismatic crystals, prismatic crystals in the lamina section (iii), multicellular glandular trichomes (iv), and papillae (Figure 2D). Further investigations included authentic plant specimens of *A. indica* (AI), and *T. crispa* (TC). The microscopic analysis of the authentic AI and TC specimens aligned with the AI and TC monographs in the Thai Herbal Pharmacopoeia (THP). Moreover, 13 unknown samples were used for quality evaluation. Photomicrographs of eight samples (Unk 02–04 and Unk 10–14) acquired from traditional drugstores corresponded with the AP monograph, whereas microscopy of two samples (Unk 01 and Unk 09) did not conform to the AP monograph. Microscopic examination of sample Unk 05 revealed polygonal epidermal cells, some containing brownish intracellular deposits, in association with bulliform cells within the upper epidermis. The lower epidermis exhibited polygonal epidermal cells, normocytic stomata, multicellular glandular trichomes, bulliform cells, and rosette aggregate calcium oxalate crystals. The coexistence of multiple diagnostic features suggests heterogeneous plant tissues, indicating that Unk 05 may represent a mixture of more than one herbal species; therefore, definitive taxonomic identification could not be concluded solely on microscopic examination. Analysis of Unk 06 and Unk 08 identified lithocysts containing cystoliths, multicellular glandular trichomes, diacytic stomata, and multicellular trichomes, consistent with the AP monograph but present in mild to moderate abundance.



(A)



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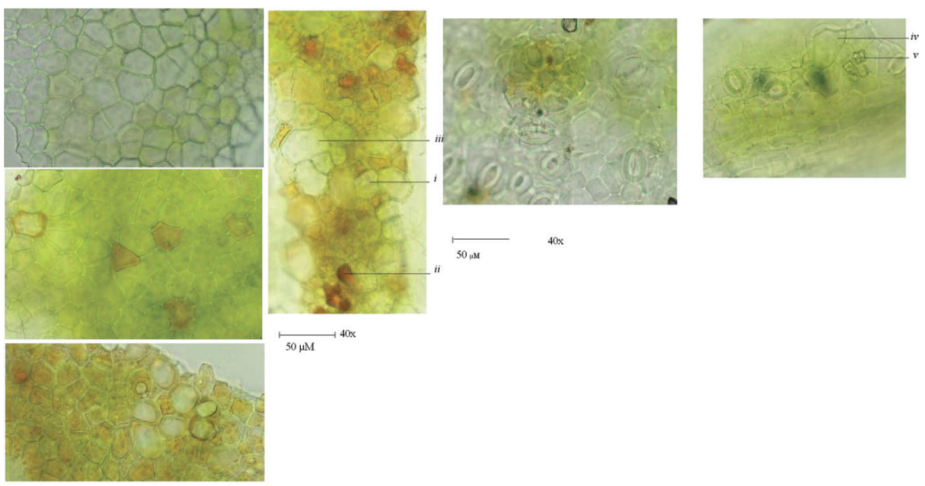

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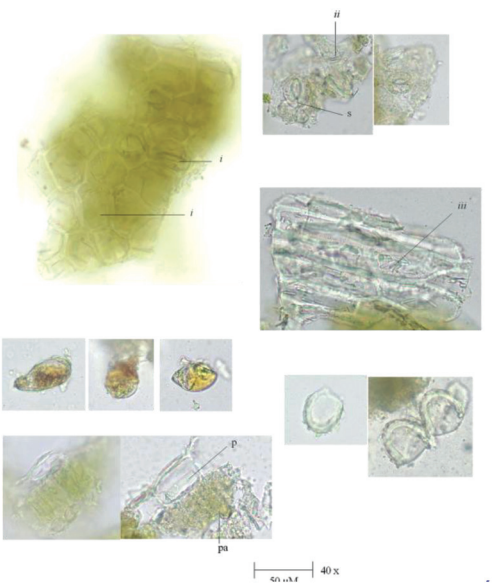

(D)

Figure 2 Microscopic photographs of powdered herbal samples and authentic plant materials were used for comparative identification. (A) Unknown sample obtained from CPPD, (B) *A. paniculata*, (C) *A. indica*, and (D) *T. crispa*.

Phytohemical Analysis Using High-Performance Thin-Layer Chromatography (HPTLC)

The HPTLC of the samples was initially undertaken by comparing their hR_F values on HPTLC and overlaying their UV spectra with those of andrographolide as a marker. HPTLC analysis was performed on 14 samples, which were separated on a silica gel GF_{254} HPTLC plate and compared with authentic specimens of AP, AI, and TC. Two chromatographic solvent systems were employed for the analysis.

As shown in Figure 3A, the chromatograms of authentic specimens of AP, AI, and TC (tracks 2–4) were compared with the unknown samples (tracks 5–18), and standard andrographolide (track 1) was used as a reference for identifying AP. After developing the plate using the solvent system I and performing derivatization, detection under white light revealed seven purple bands at hR_F 8, 10, 25, 28, 56, 61, and 87; a pink band at hR_F 42; and two brown bands at hR_F 78 and 95 in the chromatogram of authentic AP (track 2). Notably, a dark purple band at hR_F 56, identified as andrographolide, was observed through comparison with the standard (track 1). The authentic AI chromatograms were observed in track 3. After derivatization, three purple bands at hR_F 3, 13, and 62; a yellow-brown band at hR_F 26; three brown bands at hR_F 70, 82, and 95; and a green band at hR_F 35 were detected. Similarly, the HPTLC profile of TC was examined in track 4, where purple bands at hR_F 3, 57, 61, and 78 and three brown bands at hR_F 86, 90, and 95 were identified.

Figure 3B illustrates the HPTLC patterns of the samples developed using solvent system II. The chromatogram of authentic AP (track 2) displayed seven purple bands at hR_F 3, 5, 25, 28, 30, 56, 62, and 87, along with two brown bands at hR_F 78 and 95. The purple band at hR_F 30 was identified as andrographolide based on its hR_F and color (track 1). The HPTLC pattern of AI (track 3) showed a greenish-brown band at hR_F 3 and a yellow-brown band at hR_F 26. Additionally, four bands at hR_F 35, 62, and 70 with green, orange, purple, and brown colors were observed and considered characteristic of AI. The HPTLC profile of TC (track 4) revealed four purple bands at hR_F 3, 57, 61, and 78, along with a grey band at hR_F 35 and three brown bands at hR_F 86, 90, and 95. This region was identified as a distinguishing feature of TC.

The chemical profiles of the suspected sample from CPPD (track 11) and other samples were analyzed and compared with the authentic specimens under the previously described conditions. The chromatogram of the suspected sample developed using solvent system I revealed four purple bands at hR_F 3, 57, 61, and 78, along with three brown bands at hR_F 86, 90, and 95. Similarly, its HPTLC profile developed with solvent system II showed four purple bands at hR_F 3, 57, 61, and 78, a grey band at hR_F 35, and three brown bands at hR_F 86, 90, and 95. Based on these results, the chromatographic profiles indicated a dissimilarity between the suspected sample and authentic AP; however, the product exhibited a pattern similar to TC.

The HPTLC profiles of the eleven unknown samples (Unk 02–06, Unk 08, Unk 10–14) as shown in tracks 6–10, 12, and 14–18 of Figure 3A were analyzed under white light following derivatization with an anisaldehyde-sulfuric acid reagent. After developing the plate using solvent system I, the chromatograms revealed seven purple bands at hR_F 8, 10, 25, 28, 56, 61, and 87, a pink band at hR_F 42, and two brown bands at hR_F 78 and 95. When solvent system II was used (Figure 3B), the chromatograms showed seven purple bands at hR_F 3, 5, 25, 28, 30, 56, and 87, along with two brown bands at hR_F 78 and 95. Notably, a dark purple band at hR_F 56 in system I and at hR_F 30 in system II was identified as andrographolide. Based on these observations, the six unknown samples

were identified as AP. However, the appearance of grey and brown bands at hR_F 35 and 70 in samples Unk 08 and Unk 10 (tracks 12 and 14) after developing the plate using solvent system II indicated potential contributions from other species. In contrast, the HPTLC profiles of two unknown samples (Unk 01 and Unk 09, tracks 5 and 13) demonstrated dissimilarities compared to authentic AP (track 2) across both solvent systems. Based on the HPTLC results, the fingerprint of the suspected sample did not conform to the authentic AP, and the andrographolide band was not observed in the suspected sample. HPTLC enables the development of unique chemical fingerprints for herbs, effectively distinguishing authentic AP from adulterants.

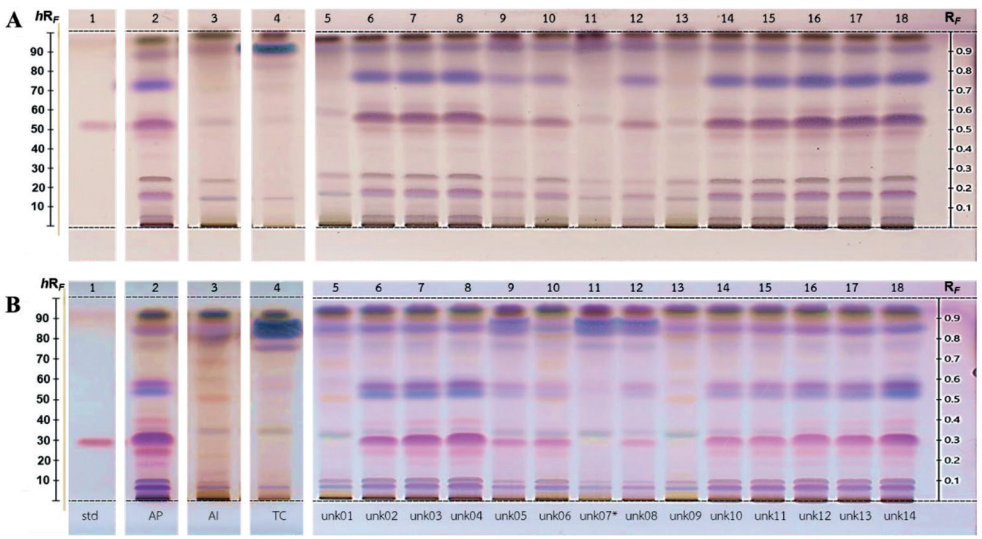

Figure 3 HPTLC chromatograms of extracts observed under white light after derivatization with anisaldehyde-sulfuric acid. The plate was developed using dichloromethane, ethyl acetate, and methanol (4:3:0.4, v/v/v) (A) and toluene: ethyl acetate in a ratio of 30:70 (v/v) (B). Tracks 1–18 represented the samples as follows: standard andrographolide (0.1 mg/mL), leaf of AP, leaf of AI, leaf of TC, and Unk 01–Unk 14, respectively. Each slot was loaded with 2 µL of the sample.

Additionally, to address this limitation of the HPTLC technique, we employed Ultra-Performance Liquid Chromatography with Photodiode Array (UPLC-PDA) for quantitative confirmation of andrographolide in selected samples following the Thai Herbal Pharmacopoeia monograph. UPLC-PDA served as a feasible alternative, providing supportive chemical evidence that aligned with HPTLC results. The specific procedures and outcomes of this analysis are presented in Table 1.

Analyzing SNPs Using MassARRAY-MALDI-TOF

Prior to SNP analysis, the species identity of the authentic plant materials (*A. paniculata*, *A. indica*, *and T. crispa*) was rigorously confirmed using DNA barcoding and sequence homology comparison. The sequence alignments showed a high degree of similarity (≥ 99%) to established NCBI GenBank accessions, confirming their purity and providing the basis for SNP marker selection (Figure 4).

A highly sensitive MassARRAY-based assay was then developed to identify and differentiate AP from adulterants AI and TC, by targeting species-specific single nucleotide polymorphisms (SNPs). The resulting mass spectral data of nucleotides complementary to the SNP positions revealed distinct Base Calls that served as definitive spectral signatures for each authenticated plant: T-T-T-C-GC-A for authentic AP, A-C-A-T-AT-A for AI, and C-T-T-T-AT-C for TC (Table 3 and Figure 4).

The MassARRAY-based assay yielded conclusive SNP profiles when analyzing the fourteen unknown samples. Six samples exhibited a single spectral signature consistent with an authentic plant species. Specifically, three samples (Unk 02–04) showed a profile identical to authentic AP, and two samples (Unk 01 and Unk 09) were identified as AI. Sample Unk 07 exhibited a profile consistent with TC, corroborating the HPTLC analysis findings. In contrast, the remaining eight samples (57.14%) were found to contain compound SNP profiles (multiple peaks) indicative of complex mixtures of two plant species (Table 3 and Figure 5). Specifically, two samples (Unk 05 and Unk 08) exhibited compound signatures matching the mixture of AP and TC, while the other six samples (Unk 06, Unk 10–14) displayed signatures confirmed to be a mixture of AI and AP.

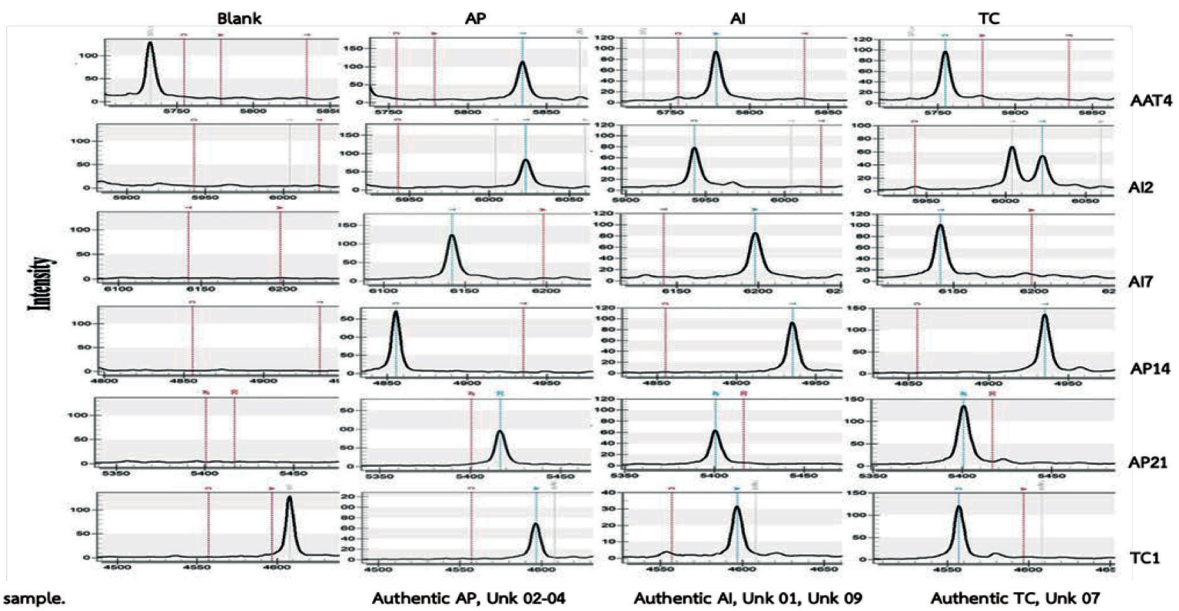

Figure 4 MassARRAY-MALDI-TOF SNP profiles of reference authentic plants and a subset of unknown samples showing single-component identification, analyzed using the iPLEX assay. The columns in the figure are labeled as follows: Blank (Negative Control); Authentic Plant References [AP (*A. paniculata*), AI (*A. indica*), and TC (*T. crispa*)]; and the specific unknown samples which are grouped according to the identified single-species profile. The rows represent the specific SNP primer sets used for the detection of the three plant species (AAT4, AI2, AT7, AP14, AP21, and TC1). Red dashed lines indicate non-extension primer products (unincorporated primers), and blue dashed lines indicate extension primer products (Base Calls). The single, clear spectral peak observed across all unknown samples confirms single-species identification without evidence of adulteration.

Table 3 The MALDI-TOF MS results in the detection of SNPs in authentic AP and 14 unknown Samples

Sample ID	Nucleotide profile						Species identification
	AAT4	AI2	AI7	AP14	AP21	TC1	
Authentic AP	T	T	T	C	GC	A	AP
Authentic AI	A	C	A	T	AT	A	AI
Authentic TC	C	T	T	T	AT	C	TC
Unk 01	A	C	A	T	AT	A	AI
Unk 02	T	T	T	C	GC	A	AP
Unk 03	T	T	T	C	GC	A	AP
Unk 04	T	T	T	C	GC	A	AP
Unk 05	T, C	T, T	T, T	C, T	GC, AT	A, C	AP, TC
Unk 06	A, T	C, T	A, T	T, C	AT, GC	A, A	AI, AP
Unk 07*	C	T	T	T	AT	C	TC
Unk 08	T, C	T, T	T, T	C, T	GC, AT	A, C	AP, TC
Unk 09	A	C	A	T	AT	A	AI
Unk 10	A, T	C, T	A, T	T, C	AT, GC	A, A	AI, AP
Unk 11	A, T	C, T	A, T	T, C	AT, GC	A, A	AI, AP
Unk 12	A, T	C, T	A, T	T, C	AT, GC	A, A	AI, AP
Unk 13	A, T	C, T	A, T	T, C	AT, GC	A, A	AI, AP
Unk 14	A, T	C, T	A, T	T, C	AT, GC	A, A	AI, AP
Blank	No call	No call	No call	No call	No call	No call	-

Note: AP = *A. paniculata*, AI = *A. indica*, TC = *T. crispa*

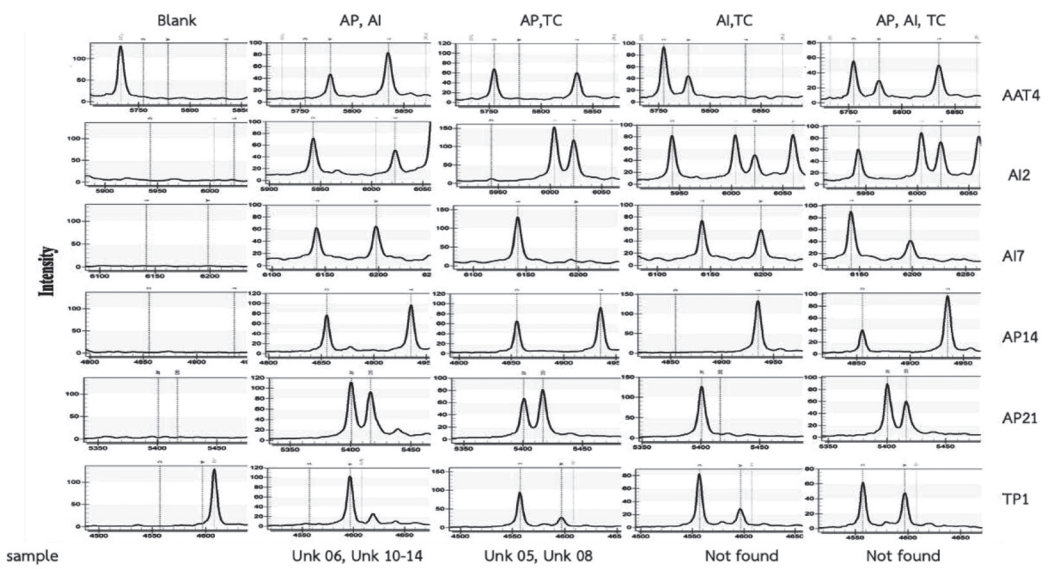

Figure 5 MassARRAY-MALDI-TOF SNP profiles of unknown samples showing compound SNP profiles indicative of adulteration, analyzed using the iPLEX sssay. The profiles reveal compound SNP signatures (multiple peaks), which confirm the co-existence of DNA from more than one plant species in a single sample, providing definitive evidence of adulteration. Columns represent the Blank (Negative Control) and the specific unknown samples, which are grouped according to the identified mixture patterns. Rows correspond to the specific SNP primer sets used for the detection of the three plant species. Red dashed lines indicate non-extension primer products, and blue dashed line indicate extension primer products.

Discussion

This study presents an integrated authentication framework combining three complementary approaches (microscopy, HPTLC fingerprinting, and MALDI-TOF MS) for the quality control of AP herbal formulations. The efficacy of the present study is not derived from the innovative use of well-established individual techniques, but from an integrative strategy that systematically addresses the limitations in each platform. Despite being the most accessible and aligned with WHO recommendations,[30-32] microscopy proved insufficient for heavily processed or powdered formulations. The comparison of the suspected samples was conducted based on macroscopic characteristics. The suspected sample from CPPD showed differences in color and taste compared to authentic *A. paniculata*. Moreover, the suspected powder exhibited a light brown color and a mildly bitter taste, which was less intense than that of AP.

The microscopic evaluation of the suspected sample was done by comparing with the Thai Herbal Pharmacopoeia (THP) monograph of AP. The suspected samples showed cellular structures indicative of a plant leaf, such as stomata and trichomes. However, the overall cellular features of the suspected capsule did not conform to the microscopic characteristics of AP in the THP. Therefore, two bitter-tasting medicinal plants, AI and TC, were selected for microscopic examination to compare their features with those of the suspected capsule. Microscopy provided unequivocal identification for raw or minimally processed samples, particularly when diagnostic features such as trichomes, starch granules, or xylem structures were preserved. However, sensitivity dropped in powdered formulations due to the loss of key anatomical markers. This highlights microscopy as a reliable yet limited tool for examining finely ground or blended herbal ingredients in polyherbal preparations. Microscopy provides essential diagnostic features of plant tissues, including trichomes, stomata, and mesophyll structures. However, its reliance on observer expertise can lead to inconsistencies, particularly when analyzing powdered samples or identical plant parts, necessitating the use of complementary techniques.[13,16]

The HPTLC technique provided chemical fingerprints with rapid throughput and reproducibility. The suspected sample from CPPD and multiple unknown samples exhibited banding patterns more closely aligned with TC, suggesting substitution or adulteration in commercial products. While HPTLC provided reproducible fingerprints and allowed discrimination between major species, it also revealed challenges. Overlapping bands, such as those at $h\mathrm{R}_\mathrm{F}$ 35 and 70, reduced specificity and suggested the presence of mixed plant materials. Furthermore, two samples (Unk 01 and Unk 09) could not be confidently identified as AP, as their chromatograms lacked the diagnostic andrographolide marker. Moreover, UPLC-PDA was used to quantitatively confirm andrographolide in the samples, following the THP monograph. This step reinforced the adaptability of the framework in resource-limited settings and highlights the importance of HPTLC validation. These findings underscore that HPTLC, though widely accepted in pharmacopoeia monographs, may not provide definitive results for heavily processed or adulterated samples. It has an advantage in generating comparative fingerprints, but its limitations highlight the need for complementary methods. In this study, HPTLC results established preliminary evidence of adulteration that required further confirmation through molecular-level techniques such as MassARRAY. While HPTLC provided reproducible fingerprints for AP and enabled preliminary discrimination from common adulterants, its specificity was reduced in complex or processed formulations. Moreover, the overlapping bands with TC and the absence

of the diagnostic andrographolide band in some samples highlight the risk of misidentification. These findings demonstrated the strength of HPTLC as a screening tool and also highlighted its limitations as a single method for authentication.

The integration of microscopy, HPTLC, and MassARRAY analyses produced highly consistent results, particularly in samples suspected of adulteration. Microscopic examination revealed the absence of key diagnostic structures of authentic *A. paniculata* (AP), such as cystoliths and diacytic stomata, in several samples. Instead, normocytic stomata and bulliform cells were observed, suggesting the presence of other plant species. These findings were supported by HPTLC analysis, which showed that the suspected samples lacked andrographolide bands (hR_F 56 or 30) and exhibited chemical fingerprints similar to *T. crispa* (TC). MassARRAY SNP profiling further confirmed adulteration in 11 of 14 samples, identifying *A. indica* (AI), TC, or mixtures of AP with these species. The strong agreement among the three analytical platforms demonstrates robust cross-validation and highlights the reliability of the integrative approach, which improved the sensitivity and specificity of adulteration detection to approximately 96–100%.[33–35]

The detection of AI and TC, both bitter-tasting herbs, raises concerns regarding raw material substitution, especially during periods of heightened demand such as the COVID-19 pandemic. Collectively, the microscopy, HPTLC, and MassARRAY results consistently indicated the presence of non-AP plant materials in commercial AP capsules, underscoring the importance of multilayered analytical strategies for quality assurance. In this workflow, MassARRAY served as the decisive confirmatory method. Unlike HPTLC, which depends on visible chromatographic bands, MassARRAY generates species-specific SNP signatures capable of distinguishing AP from adulterants even in powdered capsule matrices, thereby resolving ambiguities arising from morphological distortion or overlapping chemical profiles.

This study recognizes an important limitation: only two commonly reported substitute species, *A. indica* (AI) and *T. crispa* (TC) were included as reference adulterants. Their selection was informed by market surveillance data, pharmacognostic resemblance to *A. paniculata* (AP), and their similarly bitter taste profiles, all of which render them plausible substitutes during periods of AP shortage. Although AI and TC represent documented adulterants in the commercial supply chain, the botanical diversity of herbal raw materials suggests that additional, uncharacterized species may also be used as substitutes. Given that the authentication markers and SNP assays were specifically optimized for AP, AI, and TC, adulteration involving species outside this reference set may evade detection, particularly when phytochemical fingerprints overlap or the targeted SNP polymorphisms are absent. Within this context, the integrated analytical workflow encompassing microscopy, HPTLC fingerprinting, and MassARRAY-based SNP genotyping demonstrated strong utility for clarifying inconclusive findings, distinguishing true from false positives, and providing mutually reinforcing evidence across morphological, chemical, and genetic levels. Minor inconsistencies observed among the methods are attributable to the inherent heterogeneity of herbal materials rather than methodological deficiencies, emphasizing the necessity of multi-tiered verification.[34,36] Overall, this framework establishes a rigorous, evidence-based model for enhancing the reliability of herbal authentication, supporting regulatory assessment, and strengthening quality control practices by linking traditional

pharmacognostic evaluation with modern analytical standards, while underscoring the need to expand reference databases and explore future incorporation of chemometric or genomic tools.[33]

Finally, the sensitivity of the integrated technique was calculated based on the three samples (Unk 02–04) classified as true AP by all methods. According to standard diagnostic definitions, the MassARRAY SNP assay achieved 100% sensitivity in this dataset when evaluated against the integrated framework. The definitions of true positive (TP), false negative (FN), and sensitivity used in this analysis are consistent with internationally accepted diagnostic metrics, such as those described by Oehr (2025) in *Diagnostics*[35]. The integration of microscopy, HPTLC, and MassARRAY represents a significant advancement in herbal product authentication and provides a scientifically rigorous model that can be adapted and expanded for future applications. A comparative summary o individual methods and the integrated workflow is presented in Table 4 to illustrate how the strengths and limitations of each technique are balanced within the combined system.

Table 4 Comparative summary of the methods of microscopy, HPTLC and MassARRAY, vs integration of the three methods

Method	Strengths	Limitations	Outcome in this study
Microscopic identification	- Rapid, low-cost - Microscopic characteristics - Aligned with Pharmacopoeia standards	- Not applicable to powders/capsules - Structural markers are often lost during processing	- Confirmed authenticity in raw samples; inconclu sive in processed formulations
HPTLC	- Reproducible phytochemical fingerprints - Widely accepted in regulatory QC	- Band overlap with related species (e.g., *T. crispa*) - Limited specificity in polyherbal powders	- Detected andrographolide in authentic AP; flagged 2 ambiguous samples
MassARRAY	- High sensitivity and specificity - Works in processed or complex mixtures	- Requires a comprehensive spectral library - Advanced instrumentation needed	- Provided unambiguous identification; resolved false positives/negatives from HPTLC
Integrated (≥ 2 or 3 techniques)	- Compensates for individual weaknesses - Cross-validated evidence - Transparent, regulator-ready	- Requires multi-platform access and coordination	- Achieved 96.4% sensitivity, 100% specificity[35-37]; created an audit trail for discrepant cases

Conclusions

This study demonstrates that integrating microscopy, HPTLC fingerprinting, and MassARRAY technology into a structured decision framework offers a robust and regulator-ready approach for authenticating *A. paniculata* herbal formulations. The strength of this work lies not only in the novelty of each method individually but also in their deliberate convergence, which mitigates methodological blind spots and ensures higher sensitivity, specificity, and reproducibility than any single technique alone. By converting disparate tools into a coherent workflow, the study advances herbal medicine quality control from practice-based judgment to a transparent, auditable, and evidence-driven process. This framework has the potential to strengthen regulatory enforcement, safeguard consumer safety, and enhance trust in herbal products deployed during public health crises. Its adoption, coupled with future expansion of spectral libraries and digital decision-support systems, could set a new benchmark for herbal product authentication worldwide.

Disclaimer

During the preparation of this work, the authors used Grammarly (Grammarly Inc., San Francisco, CA, USA) to improve readability and eliminate grammatical errors. Subsequently, the manuscript underwent professional English editing by Enago (NJ, USA). The authors reviewed and edited the content after using these services and take full responsibility for the content of the publication. Furthermore, minor editing was done by a DMSc Journal advisor, as per the WHO style guide and the Standards for transcribing the Thai alphabet into the Roman alphabet by conveying the sound of the Royal Institute of Thailand, 1999.

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Comparison of Techniques for Identification of Plant Adulteration in *Andrographis paniculata* (Burm.f.) Nees Herbal Products among Microscopy, High-Performance Thin-Layer Chromatography, and MassARRAY-Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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ABSTRACT The COVID-19 pandemic between 2019 and 2021 led to an increased demand for *Andrographis paniculata* (Burm.f.) Nees (AP) products, resulting in rising prices and adulteration of products with other herbal materials. This study aimed to authenticate *A. paniculata* capsule products. Thirteen samples were purchased from traditional drugstores and local herbal shops, and one product was obtained from the Consumer Protection Police Division (CPPD). Multiple authentication techniques were applied, comprising microscopic identification, high-performance thin-layer chromatography (HPTLC) analysis, and single nucleotide polymorphism (SNP) identification using the MassARRAY-MALDI-TOF. Microscopic examination confirmed that 11 samples did not contain *A. paniculata*. The HPTLC method was used to detect andrographolide, a marker compound characteristic of this herb. The results showed that 11 samples exhibited chromatographic profiles consistent with authentic *A. paniculata*, while two samples and one sample obtained from CPPD lacked andrographolide. The MassARRAY analysis further revealed adulteration in 11 samples, identifying three as authentic *A. paniculata*, two as *Azadirachta indica* A. Juss, eight as mixtures of *A. paniculata*, *A. indica*, and *Tinospora crispa* (L.) Miers ex Hook.f. & Thomson, and the sample from CPPD as *T. crispa*. This study indicates that although HPTLC is

suitable for preliminary chemical screening, the MassARRAY technique provides higher sensitivity and specificity for confirming herbal authenticity. The integration of these three analytical approaches (microscopy, HPTLC, and MassARRAY) enhances the reliability of species authentication and supports consumer protection against adulterated *A. paniculata* products.

Keywords: *Andrographis paniculata*, Adulteration in herbal product, Microscopic identification, HPTLC, MassARRAY