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Establishment of Thin Layer Chromatographic Fingerprints for the Quality Control of Chanthalila Preparation, A Thai Traditional Antipyretic Medicine

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Abstract:

Objective: This study aimed to develop thin layer chromatographic (TLC) fingerprints to control the quality of Chanthalila preparation (CP).

Material and Methods: Twelve batches of reference CP were prepared from various sources and used as reference batches (CP1 to CP12). Artemisinin, atractylodin, eurycomanone, imperatorin and loureirin A were used as markers. The common and characteristic bands were assigned using the reference TLC fingerprints and applied to assess the qualitative parameters used in the identification and chemical profiling of eight commercial CPs.

Results: The reference TLC fingerprints were established on a silica gel GF254 plate with two mobile phase systems, *System A* and *System B*. In *System A*, atractylodin, imperatorin, and loureirin A were assigned as characteristic bands along with 10–12 common bands under Ultraviolet (UV) 254 nm, UV 366 nanometer (nm), and derivatization. In *System B*, 6 common bands were observed under UV 254 nm, while 11 fluorescent bands were detected under UV 366 nm. Eurycomanone and artemisinin were not found in any reference CP batches, which revealed the TLC method had poor sensitivity. The TLC patterns of commercial CPs were markedly different from those of the reference fingerprints. Most commercial products failed to meet the specification criteria because only imperatorin appeared in the chromatogram.

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Conclusion: TLC fingerprinting is a fast and efficient method that can be employed in the quality control of CP. It enables the revelation of the quality of the raw materials in the chromatograms, which can be useful to manufacturers to evaluate their supplier sources. However, the sensitivity of this method for determining some markers was quite low.

Keywords: Chantaleela, Chanthalila, combinative herbal preparation, TLC fingerprints, traditional medicine

Introduction

Chanthalila or Chantaleela preparation (CP) is an antipyretic Thai traditional medicine that has been approved by the National List of Essential Medicines of Thailand[1]. This combinative herbal preparation consists of eight medicinal herbs (*Angelica dahurica* root, *Artemisia annua* aerial parts, *Atractylodes lancea* rhizome, *Dracaena cochinchinensis* reddish fungal-infected wood, *Eurycoma longifolia* root, *Gymnopetalum chinense* fruit, *Santalum album* heartwood, and *Tinospora crispa* stem) and is flavored with borneol. CP is widely used in hospitals and has been launched successfully on the market as well. CP has been found to have various pharmacological activities, both *in vivo* and *in vitro*, that are related to its anti-fever activity, including antipyretic, anti-inflammatory, analgesic, and anti-ulcerogenic effects[2]. The treatment efficiency of multiple-component herbal preparations depends on the quality of their raw materials, which fluctuates depending on geographical source, climate, cultivation conditions, harvest time, drying process, and storage[3–6]. Moreover, preparation procedures, inclusive of coincidental or deliberate adulterations, directly affect their therapeutic effectiveness[5,7]. Quantitative analysis using a few markers is not comprehensive for the overall quality control of the complex chemical components in combinative medicine[5,8].

The challenges of quality control and identification in multiple-component herbal preparations relate to the analytical methods that can determine many substances simultaneously. Nowadays, the chromatographic fingerprinting technique is commonly used since it is able to separate chemical compounds and perform a characteristic profile reflecting the complex chemical constituents in the sample[9]. The World Health Organization (WHO) and other regulatory bodies recommend chromatographic fingerprint analysis for the assessment of natural products or herbal medicines[7,10–11]. There are various techniques used in fingerprint analysis; the most popular are thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). TLC is a readily available technique used in the fast screening of samples to identify herbal materials. Its advantages are that it is easy to use, it enables the simultaneous analysis of multiple samples, and it is an economical technique requiring few quantities of solvents. One of its significant advantages is its flexibility to optimize operational parameters (e.g., plate development, sample application, documentation, and derivatization).

Our previous work developed an effective HPLC fingerprint for the quality control of CPs[12]. It was found that the HPLC system was not appropriate for screening or analyzing large numbers of samples. The HPLC system was able to analyze only one sample at a time, and the processing time was about 2 hours for each sample. Even though the TLC fingerprint of this preparation was addressed in the monograph that was officially published in the Thai Herbal Preparation Pharmacopoeia (THPP)[13], the reference fingerprint did not specify any marker compounds, which is one of the most important aspects of fingerprint development. In this study, a TLC fingerprint of CP was developed for both polar and non-polar systems in order to assess the overall quality of the preparation. Standard markers were used to identify significant bands, and the commercial CPs were also challenged using the established fingerprints.

Material and Methods

Chemicals, solvents, and reagents

The reference standards of artemisinin (99.30%), eurycomanone (95.79%), and loureirin A (99.43%) were purchased from Chengdu Biopurify Phytochemicals (Chengdu, China). Atractylodin (99.89%) and imperatorin (99.27%) were purchased from Chengdu Must Biotechnology (Chengdu, China).

The analytical grade solvents of dichloromethane (DCM), ethanol (EtOH), ethyl acetate (EtOAc), glacial acetic acid (GAA), hexane, isopropanol (iPrOH), methanol (MeOH), sulfuric acid (SA), and toluene were purchased from RCI Labscan Ltd., Thailand. Meanwhile, the analytical grade reagents of anisaldehyde or 4-methoxybenzaldehyde (Alfa Aesar, UK), diphenylboric acid-2-aminoethyl ester (Fluka, Germany), and polyethylene glycol (KemAus, Australia) were purchased from the specified companies. Distilled water was used in the Elga water purification systems.

The derivatizing reagent of 10% v/v of SA in EtOH was prepared by carefully adding 10 mL of SA to 80 mL of EtOH, then adjusting to 100 mL with EtOH. Anisaldehyde test solution (TS) was prepared by mixing in order, 0.5 mL of anisaldehyde with 10 mL of GAA, followed by 85 mL of MeOH. The natural product (NP) TS was prepared by dissolving 1 g of diphenylboric acid-2-aminoethyl ester in sufficient MeOH to make 100 mL. The polyethylene glycol (PEG) TS was prepared by dissolving 5 g of PEG-4000 in sufficient EtOH to make 100 mL.

Instrumentation

The TLC system (Camag, Muttenz, Switzerland) consisted of a Linomat-5 sample applicator equipped with a 100-µL sample dosage syringe, twin trough chambers (20×10 cm and 20×20 cm), a TLC visualizer-2, and a TLC plate heater-III. The aluminum TLC plates pre-coated with silica gel 60 GF_{254} (20×20 cm) were purchased from Merck (Darm-stadt, Germany).

Herbal materials

Each plant material, i.e., *A. dahurica*, *A. annua*, *A. lancea*, *D. cochinchinensis*, *E. longifolia*, *G. chinense*, *S. album*, and *T. crispa*, was collected or purchased from 6 different sources to ensure the representation of quality variability among the different raw materials. The authenticity of the plant species was determined by comparing them with the herbarium specimens in the Faculty of Pharmaceutical Sciences at Prince of Songkla University. The quality of each plant material was assessed in accordance with their mandatory compliance with the Thai Herbal Pharmacopoeia (THP) specifications[14]. The standardized materials were cleaned, dried at 50-60°C for 12 h, pulverized to a fine powder, and then passed through a number-60 sieve. Borneol was purchased from a herbal drug store and assessed for quality according to the Chinese Pharmacopoeia stipulations[15].

Preparation of CP reference batches

Twelve reference batches of CP were prepared in compliance with the National List of Essential Medicines.[1] Each plant material (60 g) was geometrically mixed with borneol (15 g) to produce 12 reference batches of CP (CP1, CP2, …, CP12). The herbal component of each reference batch was selected via randomly sampling without replacement in order to create a variation of the reference batches. The preparations were kept in an airtight container and protected from light until used.

Preparation of standard solution

A mixed-standard solution of artemisinin, atractylodin, eurycomanone, imperatorin, and loureirin A was prepared in MeOH at a concentration of 0.5 mg/mL of each compound, except for atractylodin that had a concentration of 0.2 mg/mL, and was used as a reference marker.

Optimization of extraction solvents and preparation of test solutions

The extraction solvents of distilled water, 50% v/v MeOH, 80% v/v MeOH, MeOH, DCM, and hexane were used in CP extraction. The most appropriate solvent was used in the further extraction of reference batches (CP1 to CP12), individual herbal components, and commercial CPs (P1 to P8). The CPs (2 g) were separately extracted in 10 mL of the extraction solvent using ultrasound-assisted extraction for 60 min. The extracts were filtered and evaporated, then reconstituted to 2 mL with the extraction solvent. The test solutions were filtered through a 0.45-µm nylon membrane filter before undergoing TLC analysis.

Optimization of TLC conditions

The TLC fingerprints of CP and each herbal component were developed using a semi-automatic TLC system. The conditions were optimized as application volumes ranged from 5 to 20 µL for a mixed-standard solution or sample solution. The separation was carried out using a TLC plate coated with silica gel GF_{254} as a stationary phase. TLC chromatograms were developed in a twin trough, vertical, glass chamber. The presence of all substances was identified by comparing band characteristics and the retardation factor (R_f) values. The most appropriate systems were selected based on their ability to clearly separate marker bands without overlapping with other bands, and the 100-fold R_f values (hR_f) ranged between 20 and 80. The TLC pattern was detected under white light, UV 254 nm, UV 366 nm, and derivatization using anisaldehyde TS followed by heating at 105°C for 10 min (Spray I) and 10% v/v SA in EtOH followed by heating at 105°C for 10 min (Spray II). The NP/PEG TS, meanwhile, was assessed under UV 366 (Spray III) to detect chemical components in CP and further establish the TLC fingerprint.

Establishment of TLC fingerprints for CP

The reference batches (CP1 to CP12) and 8 herbal components were analyzed under the optimized TLC conditions. Critical parameters for identification and qualitative analysis, including common and characteristic bands, were established from the data obtained from the 12 fingerprints. The bands that were present in almost all reference samples were considered common bands. The bands that corresponded to markers were identified as characteristic bands. The R_f and hR_f values of the common and characteristic bands were calculated as follows:

$$R_f = \frac{\text{Distance of each band}}{\text{Distance of solvent front}}$$

$$hR_f = R_f \text{ of each band} * 100$$

The TLC fingerprints of CP were established by listing the hR_f values of the common and characteristic bands. The band characteristics under white light, UV, and derivatization were also recorded in the fingerprint.

Quality assessment of commercial CPs using the developed TLC method

Eight commercial CPs from different brands (P1 to P8) were prepared and analyzed using the developed TLC method. The similarity of TLC patterns among samples and reference fingerprints was evaluated. In order to meet the specifications to be include in the fingerprint, all characteristic bands must had been observable and not less than 80% of the common bands needed to be found in the tested chromatogram.

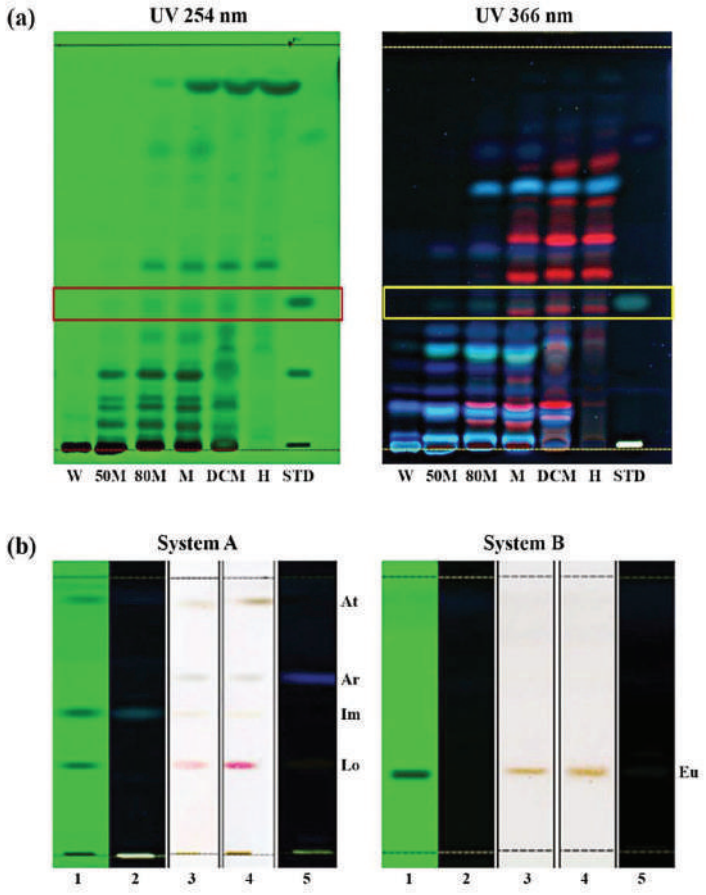
Results and Discussion

Development of TLC conditions

The chemical markers of CP were selected according to each herbal component. Artemisinin, atractylodin, eurycomanone, imperatorin and loureirin A were considered as the standard markers to represent the quality of *A. annua*, *A. lancea*, *E. longifolia*, *A. dahurica*, and *D. cochinchinensis*, respectively. Our previous work found that the level of

apigenin, the marker of *T. crispa*, in the preparation was low while α-santalol, the marker for *S. album*, was not available commercially[12]. Thus, apigenin and α-santalol were excluded from the mixture of standard markers.

In this study, there were 2 mobile phase systems used to detect the chemical components in the CP extract. *System A* consisted of a mixture of hexane 70: acetone 20: EtOAc 10, which allowed the solvent front to reach 13 cm. Atractylodin, imperatorin, and loureirin A were detected via quenching under UV 254 nm, and only imperatorin was detected under UV 366 nm (green fluorescent). Derivatization with Spray I revealed atractylodin (brown color), loureirin A (red-pink color), imperatorin, and artemisinin (pale color); meanwhile, derivatization with Spray II was able to detect artemisinin more clearly under UV 366 nm (blue fluorescent), as shown in Figure 1. Due to



TLC = Thin layer chromatography, CP=Chantalila preparation, W=water extract of Chantililla preparation, 50M= 50% methanolic extract of Chantililla preparation, 80M=80% methanolic extract of Chantililla preparation, M=methanolic extract of Chantililla preparation, DCM=dichloromethane extract of Chantililla preparation, H=hexane extract of Chantililla preparation, STD=standard, At=atractylodin, Ar=artemisinin, Im=imperatorin, Eu=eurycomanone, Lo=loureirin A, UV=ultraviolet, nm=nanometer

Figure 1 (a) TLC patterns of CP extraction with water (W), 50% methanol (50M), 80% methanol (80M), methanol (M), dichloromethane (DCM), and hexane (H); and the standard markers (STD) in *System A* detected under UV 254 nm and UV 366 nm. (b) Bands of standard markers—atractylodin (At), artemisinin (Ar), imperatorin (Im), loureirin A (Lo), and eurycomanone (Eu)—in *System A* and *System B* detected under UV 254 nm (1), UV 366 nm (2), white light after Spray I (3), white light after Spray II (4), and UV 366 nm after Spray II (5)

the huge differences in polarity between markers, *System A* was unable to isolate eurycomanone from the other markers. *System B* was performed to detect the presence of eurycomanone in CP. The mixture of the mobile phase in *System B* was toluene 6: iPrOH 1.5: EtOAc 3: MeOH 2: water 0.3, which allowed the solvent front to reach 8 cm. The results showed that *System B* was suitable for the detection of only eurycomanone. The quenching band at UV 254 nm and both derivatizations were related to eurycomanone, as shown in Figure 1.

Optimization of extraction solvents

The extraction solvent is a key factor in extraction efficiency[5]. The CP water extract gave the lowest variety of compounds and was not suitable for normal phase chromatographic separation. Considering the extraction efficiency of marker compounds, MeOH and 80% v/v MeOH were capable of extracting three markers from CPs, while DCM and hexane were not suitable for extracting loureirin A. The methanolic extract showed a red fluorescent band that represented chlorophyll-like compounds and interfered with the imperatorin band. In contrast, the proportion of the 80% v/v MeOH extract showed a higher-clarity band without interference from red fluorescence at long wavelengths. Our results suggested that extraction with 80% v/v MeOH was the most appropriate solvent system for CP, as shown in Figure 1. Recently, THPP announced the official TLC fingerprint of CP, which employed 80% v/v EtOH as a solvent for extraction[13]. Our preliminary study found that extracting CP with MeOH showed greater reliability and consistency in terms of extraction than EtOH. Extraction with 80% v/v MeOH was associated with a larger variety of compounds in the chromatogram, rendering it better suited to evaluate the quality of CP than 80% v/v EtOH.

Establishment of TLC fingerprints

Based on the chromatographic patterns obtained from the 12 reference batches, the TLC reference fingerprints were established. Common bands were assigned to the band with the same hR_f value and similar characteristics that existed in at least 10 reference batches. The common bands that corresponded to marker compounds or major compounds were specified as characteristic bands. The hR_f values of all common bands in both *System A* and *System B*, and the band characteristics are summarized in Table 1.

Separation using *System A* yielded 10 common bands and 3 characteristic bands that corresponded to loureirin A, imperatorin, and atractylodin when observed under UV 254 nm (Figure 2a). Detection under UV 366 nm revealed 10 common bands and a band corresponding to imperatorin (Figure 2b). Both derivatizations with Spray I and Spray II assessed under white light exhibited 12 common bands with 2 characteristic bands corresponding to loureirin A and atractylodin (Figure 2c, 2d). Derivatization with Spray III evaluated under UV 366 nm, displayed 10 common bands with 2 characteristic bands corresponding to imperatorin and atractylodin (Figure 2f). In *System B*, the TLC fingerprints viewed under UV 254 nm and 366 nm resulted in 6 and 11 common bands, respectively. However, eurycomanone was not detected in any reference batch (Figure 3a, 3b).

Considering the chemical constituents of the CP fingerprint as well as the CP herbal components in *System A*, it was found that most of the bands evidenced in the CP fingerprint corresponded with the bands found in each herbal component. Artemisinin was detected as a fluorescent band under UV 366 nm after derivatization with Spray II, and it was found only in the *A. annua* herbal material but not in the preparation (yellow arrow in Figure 2e). Interestingly, the band at hR_f values 87-89 under UV 254 nm (Figure 2a) was found only in CP and did not correspond to any herb component. The isolation and identification of this compound must be further investigated. The appearance of artifacts or novel compounds in multicomponent herbal preparations has been reported. Derivatives of compound D from *Zingiber cassumunar* and fatty acids from *Nigella sativa* have been reported when compounding the Prasaplai preparation[16].

Table 1 hR_f values of components in methanolic extract of CP in *System A* and *System B*

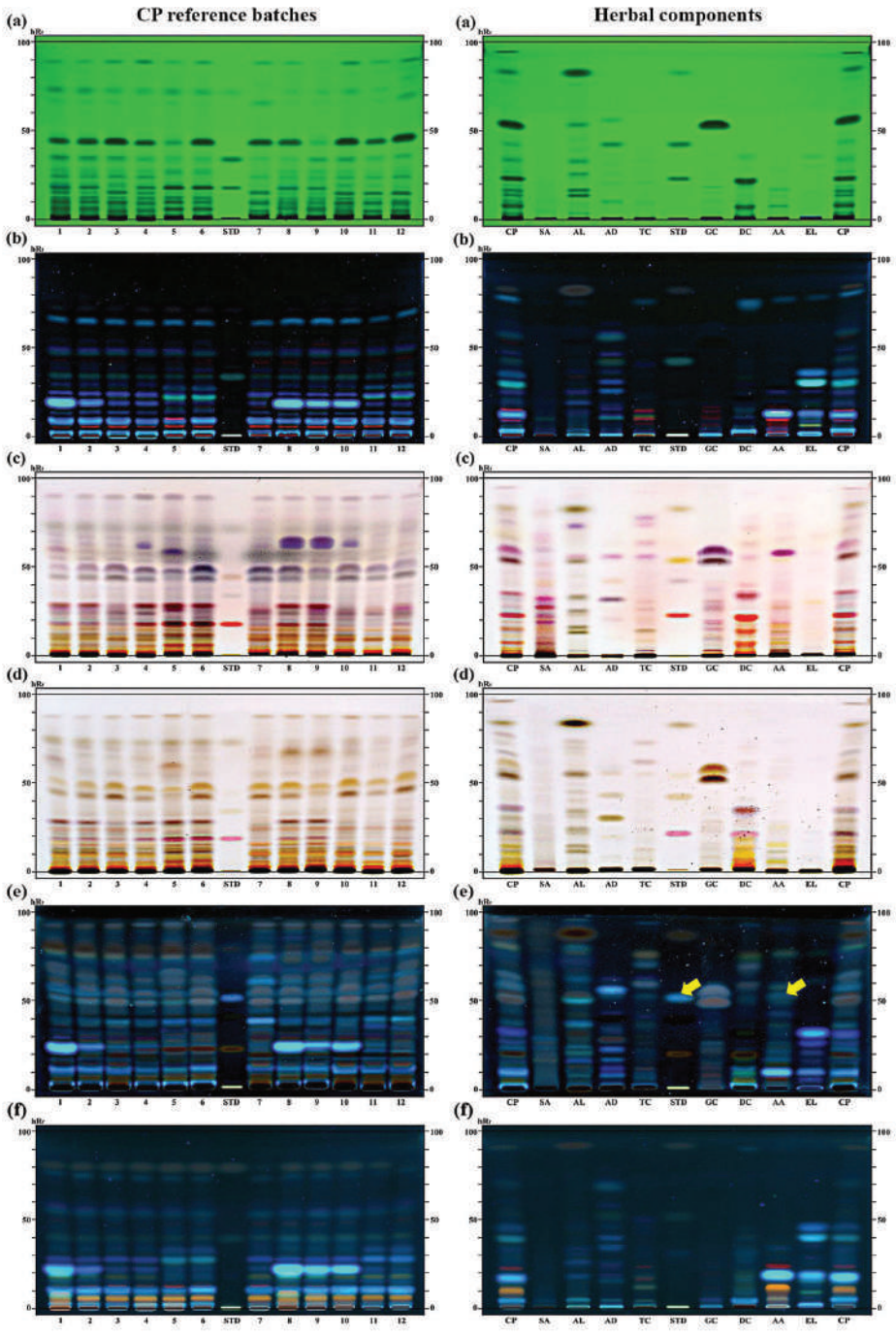
hR_f value	System A					System B	
	1	2	3	4	5	1	2
87–89	q	–	purple	brown	–	–	cyan
79–82	–	–	–	–	–	q	blue
72–75	q[At]	–	brown[At]	brown[At]	brown[At]	q	blue
67–69	–	–	purple	brown	pale cyan	–	red
64–66	–	cyan	violet	brown	–	–	–
63–64	–	–	–	–	–	q	cyan
59–62	–	–	–	–	pale blue	q	cyan
53–55	–	–	–	–	pale cyan	q	–
51–53	–	–	–	–	–	q	cyan
49–50	–	blue	purple	brown–yellow	–	–	–
46–48	–	green	–	–	–	–	–
43–44	q	–	dark purple	dark brown	–	–	–
34–36	q[Im]	green[Im]	–	–	green[Im]	–	–
28–30	–	–	red	red–brown	pale cyan	–	–
26–28	q	–	–	–	blue/green	–	–
23–25	q	blue	–	–	–	–	cyan
22–23	–	–	–	–	–	–	green
18–19	q[Lo]	blue	red[Lo]	pink[Lo]	–	–	cyan
12–13	q	pale cyan	–	–	–	–	–
10–12	–	–	orange	brown	–	–	green
9–10	q	cyan	orange	brown	cyan	–	–
5–6	q	red	orange	orange	yellow	–	–
2–3	–	cyan	orange	orange	cyan	–	–

1: UV 254 nm, 2: UV 366 nm, 3: white light after Spray I, 4: white light after Spray II, 5: UV 366 nm after Spray III
q=quenching, Lo, Im, and at refer to the marker compounds loureirin A, imperatorin, and atractylodin, re-spectively
CP=Chantalila preparation, At=atractylodin, Ar=artemisinin, Im=imperatorin, Eu=eurycomanone, Lo=loureirin A, UV=ultraviolet

Although eurycomanone was not appearent under UV 254 nm, *System B* was able to detect the presence of *E. longifolia* under UV 366 nm (Figure 3b).

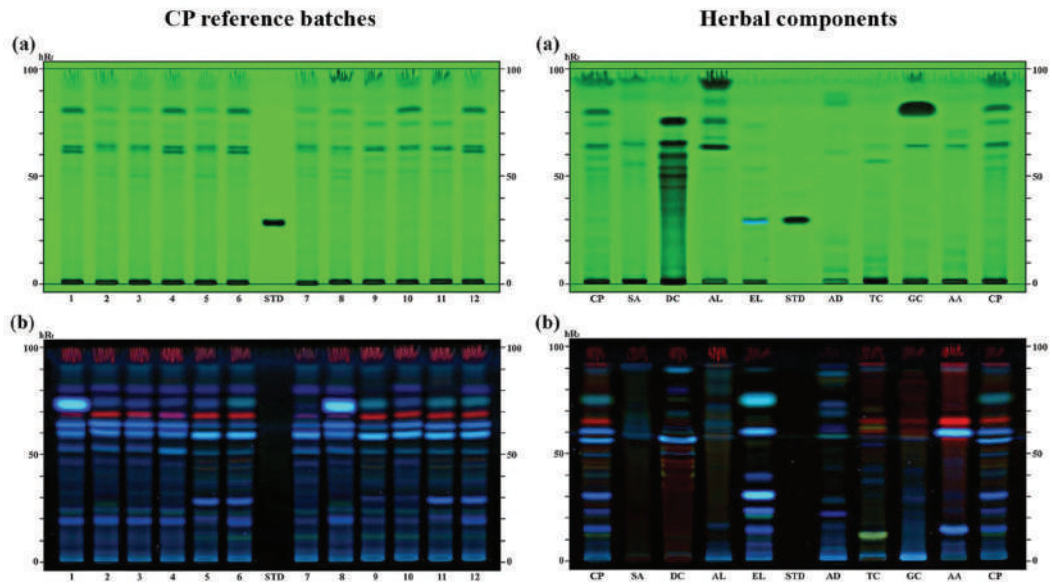
Our results implied that TLC is a simple and useful tool for the qualitative analysis of multiple samples simultaneously. However, it suffers from limitations related to its low sensitivity and the detection of low-content compounds. The reference TLC fingerprints demonstrated high resolution bands corresponding to atractylodin, imperatorin, and loureirin A. The bands representing artemisinin and eurycomanone, on the other hand, were of a low resolution; they appeared as a pale quenching or colorization, making them difficult to identify. This finding agrees with those of another study on the HPLC fingerprint analysis of CP, which detected a low content of eurycomanone[12]. As a consequence of the presence of many different compounds in CP, it is impossible to analyze all markers using one system; to do so, both polar and non-polar systems would be required. *System A* was the most suitable method for developing the TLC fingerprint of CP. Meanwhile, *System B* was used to complete the quality assessment.

In respect to the TLC patterns of CP compared with those of its herbal components, hR_f might not clearly provide corroborative evidence for the identification of complex plant samples because different compounds may possibly have the same R_f value[17] or the same substance can be found in many herbals. When comparing our TLC system with the officially published fingerprint in THPP[13], it was found that our system



TLC=Thin layer chromatography, CP=Chantalila preparation, STD=standard, SA=S. album, AL=A. lancea, AD=A. dahurica, TC=T. crispa, GC=G. chinese, DC=D. cochinchinensis, AA=A. annua, EL=E. longifolia, nm=nanometer

Figure 2 TLC patterns of 12 CP reference batches and the herbal components of *S. album* (SA), *A. lancea* (AL), *A. dahurica* (AD), *T. crispa* (TC), *G. chinense* (GC), *D. cochinchinensis* (DC), *A. annua* (AA), and *E. longifolia* (EL) detected under (a) UV 254 nm, (b) UV 366 nm, (c) white light after Spray I, (d) white light after Spray II, (e) UV 366 nm after Spray II, and (f) UV 366 nm after Spray III using *System A*



TLC=Thin layer chromatography, CP=Chantalila preparation, UV=Ultraviolet, nm=nanometer, P1=commercial Chantalil products 1, P2=commercial Chantalil products 2, P3=commercial Chantalil products 3, P4=commercial Chantalil products 4,P5=commercial Chantalil products 5, P6=commercial Chantalil products 6, P7=commercial Chantalil products 7, P8=commercial Chantalil products 8

Figure 3 TLC patterns of 12 CP reference batches and herbal components *S. album* (SA), *A. lancea* (AL), *A. dahurica* (AD), *T. crispa* (TC), *G. chinense* (GC), *D. cochinchinensis* (DC), *A. annua* (AA), and *E. longifolia* (EL) assessed under (a) UV 254 nm and (b) UV 366 nm using *System B*

(*System A*) resulted in clearer separation and superior resolution in relation to imperatorin and the major band of *G. chinense* than the officially published chromatogram (Mobile phase 1). The derivatization with 10% v/v sulfuric acid and the subsequent assessment under UV 366 nm exhibited a fluorescent band corresponding to artemisinin, the active constituent of *A. annua*, while the official chromatogram did not use this derivatizing agent. Moreover, in comparison to mobile phase 2 (THPP), *System B* (our system) achieved a clearer separation of the polar compound bands, especially during evaluation under UV 366 nm.

Quality assessment of commercial CP products using the developed TLC method

Commercial CPs were purchased, and their quality was evaluated using the developed method and compared with the CP reference fingerprints. Most of the products showed slightly different patterns from the reference fingerprints, which implied inconsistencies in the raw material quality. The extraction problem encountered with P8 may be related to the excipients of the tablet. Imperatorin appeared in all products, while atractylodin and loureirin A were undetectable (Figure 4). The band of interest at an hR_f value of 87–89 under UV 254 nm using *System A* was not found in any product. According to the results, the commercial CPs contained low quantities of some compounds as well as misidentified herbal components in the preparation. The results of the *System A* analysis, involving derivatization with Spray III and detection under UV 366 nm, revealed that the yellow fluorescent bands, which are related to *A. annua*, were absent. Moreover, foreign green fluorescent bands were found (Figure 4e), which might indicate an improper usage of plant materials, especially *A. annua*, in the commercial CPs. This finding confirms our previous report that most commercial CPs use *A. vulgaris* instead of *A. annua* in their preparations[12]. In the quality control of

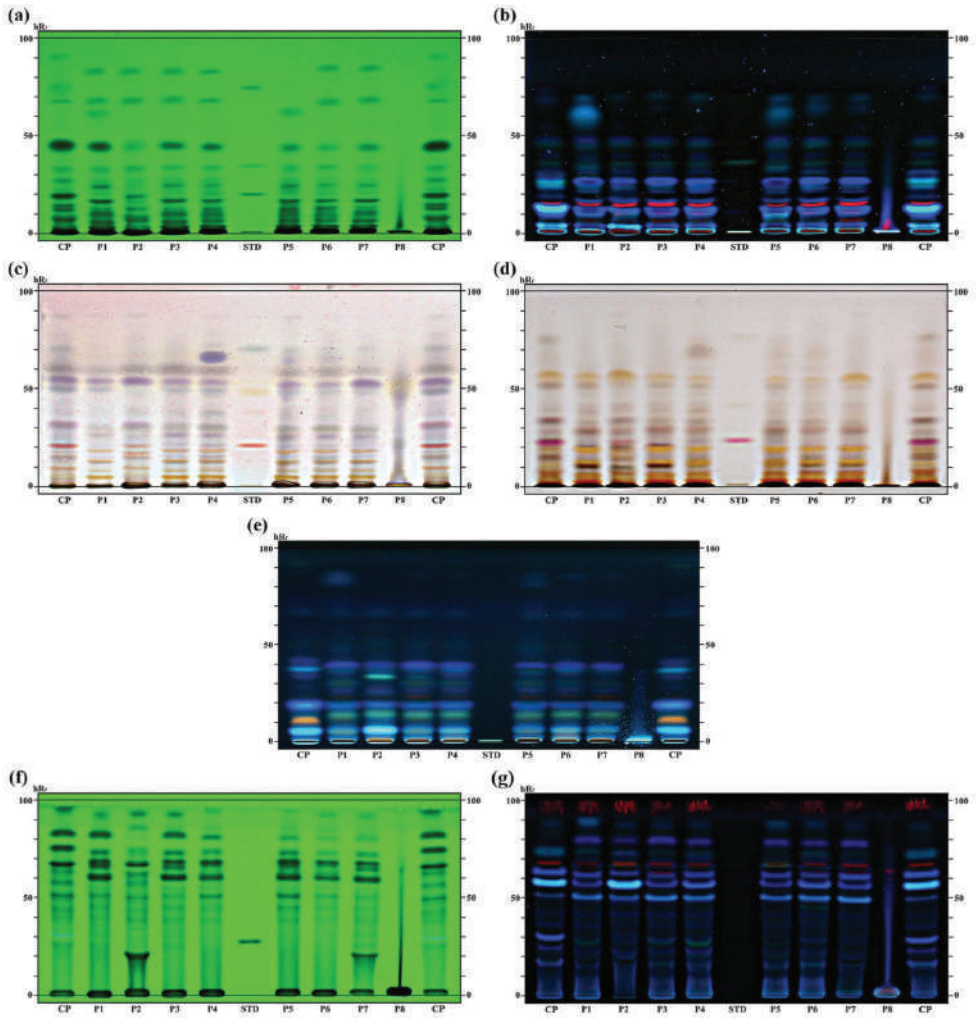


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Figure 4 TLC patterns obtained from commercial CP products (P1 to P8) compared with the CP reference fingerprint: (a) *System A* under UV 254 nm, (b) *System A* under UV 366 nm, (c) *System A* under white light after Spray I, (d) *System A* under white light after Spray II, (e) *System A* under UV 366 nm after Spray III, (f) *System B* under UV 254 nm, and (g) *System B* under UV 366 nm

herbal medicines, identifying the components of combinative herbal preparations is very important. In practice, TLC analysis is used to screen the preliminary accuracy. This means that common bands should correspond to not less than 80% of the TLC reference fingerprints and that the analysis of all characteristic bands that correspond to markers should be performed.

Conclusion

The TLC fingerprints developed in this study are useful in the screening of the chemical patterns of CPs, the efficacy of which is influenced by the quality of the raw materials it contains. These fingerprints are suitable for both herbal medicine manufacturers and quality control laboratories. Nevertheless, a significant limitation is their

low sensitivity. In summary, TLC is a fast method for the quality control of finished products, which has the capability of assessing the quality of multicomponent preparations. However, the sensitivity of this method is one of its drawbacks when compared with HPLC.

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Conflict of interest

The authors declare no conflict of interest.

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