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Original Article

Development of combinative method using HPLC fingerprints and quantitative analysis for quality assessment of Chantaleela preparation

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Abstract

Chantaleela (CTL) is a Thai traditional medicine composed of 8 medicinal herbs and borneol and used to treat fever. This study aimed to control the quality of CTL products by establishing a simple but comprehensive method using high performance liquid chromatography (HPLC). HPLC fingerprints of CTL were constructed for assessing qualitative parameters for identification and chemical profiling. This method is highly precise and accurate in quantifying the constituents of CTL identifying markers including eurycomanone, loureirin A, imperatorin and atractylodin. The quality of commercial CTL products in Thailand was assessed using this developed method. The chromatographic pattern was noticeably different from the established reference fingerprints. The substitution of *Artemisia vulgaris* for *Artemisia annua* was found in every commercial product. Moreover, the low content of marker compounds reflected poor quality of the commercial products.

Keywords: Chantaleela, HPLC fingerprint, quality control, traditional medicine, combinative herbal preparation

1. Introduction

Combinative herbal preparations are often found in traditional medicines. These combinations work to both exert curative effects and decrease undesirable effects of some herbs (Jiang, David, Tu, & Barbin, 2010). Typically, herbal preparations contain hundreds of compounds (Xie *et al.*, 2007), and their quality varies depending on geographical source, climate, cultivation conditions, harvest time, and storage. These variations affect directly efficacy and safety of a herbal medicine (Li *et al.*, 2010). Unlike modern medicine, quantitative analysis of a few markers in the preparations is insufficient to verify the preparation quality. The World Health Organization (WHO) recommends chromatographic fingerprint analysis for assessing the quality of herbal medicines. Fingerprints demonstrate some characteristic profiles and reflect the complex chemical constituents of the formulation (Goodarzi, Russell, & Heyden, 2013; Tistaert *et al.*, 2011). A combination of chromatographic fingerprint and quantitative analysis might completely characterize the quality aspects as regards component identification and treatment efficacy. HPLC has been used to control the quality of many traditional Chinese medicines (Jiang *et al.*, 2010; Yi *et al.*, 2011).

CTL is an antipyretic drug in Thai national drug list. It is composed of 8 medicinal herbs namely *Eurycoma longifolia*, *Santalum album*, *Artemisia annua*, *Tinospora crispa*, *Angelica dahurica*, *Gymnopetalum chinense*, *Atractylodes lancea* and *Dracaena cochinchinensis*, and flavored with borneol (National Committee on Drug System Development, 2019). However, CTL has faced quality issues and reliable quality control methods are lacking. As part of our interest in quality control of CTL, HPLC fingerprints and quantitative analysis that meet the qualitative and quantitative aspects of quality control were developed in this study.

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2. Materials and Methods

2.1 Reference standards

Eurycomanone (95.79%), artemisinin (99.30%) and loureirin A (99.43%) were purchased from Chengdu Biopurify Phytochemicals (Chengdu, China). Apigenin (98.03%), imperatorin (99.27%) and atractylodin (99.89%) were purchased from Chengdu Must Biotechnology (Chengdu, China). Paracetamol (99.84%) was purchased from the Department of Medical Sciences (DMSc, Thailand).

2.2 Raw materials and preparation of reference batches

Each herbal material was collected or purchased from 6 different sources to represent the quality variations in the raw materials. The plant species were authenticated and the quality had to comply with the criteria stated in the 2019 Thai Herbal Pharmacopoeia (Department of Medical Sciences, 2019). Borneol was assessed according to the 2010 Chinese pharmacopoeia (Chinese Pharmacopoeia Commission [ChP], 2010). All herbal materials were cleaned, dried at 50-60°C, pulverized to fine powder and then passed through sieve no. 60. Each herbal material (60 g) was geometrically mixed with borneol (15 g) to produce 12 reference batches of CTL (CTL1 to CTL12). The herbal component was randomly sampled without replacement to assess variations in the reference batches. The preparations were kept in airtight containers and protected from light until use.

2.3 Preparation of mixed-standard solution and internal standard solution

A mixed-standard solution consisting of eurycomanone, artemisinin, apigenin, loureirin A, imperatorin and atractylodin was prepared in MeOH to the concentration of 0.2 mg/mL for each standard marker. An internal standard (IS) solution was prepared in MeOH containing 1 mg/mL of paracetamol.

2.4 Optimization of extraction solvents and preparation of CTL extracts

CTL or an individual herbal component (2 g) was separately extracted in 10 mL of extraction solvents using ultrasound assisted extraction for 60 min. The extracts were filtered and the volumes were adjusted to 10 mL. The extract solutions were filtered through 0.22 µm nylon membrane filter. IS solution (50 µL) was added to each extract solution (1 mL) prior to being subjected to HPLC analysis.

Various extraction solvents including hexane, dichloromethane (DCM), EtOAc, MeOH and water were used for extracting CTL. Proportions of MeOH and water for CTL extraction were further studied using MeOH, 80%v/v MeOH, and 50%v/v MeOH. The most appropriate solvent was used for further extraction of reference batches (CTL1 to CTL12), individual herbal components, and commercial CTL products (P1 to P8).

2.5 Chromatographic conditions

Qualitative and quantitative analysis of CTL was carried out using the quaternary pump HPLC system (LC-20AD, Shimadzu®, Japan) equipped with a diode array detector. The separation was performed on a C18 column (ODS-80Ts, 150-mm×4.6-mm i.d. with 5 µm particle size, TSKgel® Tosoh, Japan) at 30 ± 2°C. The mobile phase was composed of 0.1%v/v aqueous formic acid and acetronitrile (ACN), and the flow rate was adjusted to 0.7 ml/min. A gradient elution was performed using the following proportions: 10% ACN in 0-8 min, 10-50% ACN in 8-60 min, 50-80% ACN in 60-75 min, 80% ACN in 75-90 min, 80-10% ACN in 90-95 min and 10% ACN in 95-100 min. Injection volume was set at 20 µL. The UV absorbance was monitored at 254 and 300 nm. The LCsolution software (Version 1.21 SP1, Shimadzu®) was used for data analysis and processing.

2.6 Validation of analytical method

The analytical method was validated to confirm the reliability of the test results. The specificity, linearity, precision, accuracy, and sensitivity were assessed for quantitative analysis (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [ICH], 2005). Meanwhile, the stability, precision and reproducibility of common peaks were evaluated as part of fingerprint analysis (Cai, Ong, & Liu, 2012).

Specificity was estimated by comparing the retention time and UV spectrum between sample extracts and standard marker solutions. Meanwhile, peak purity of markers in the sample was confirmed.

Linearity of the calibration curve of each standard marker, run at 6 concentration levels, was constructed by plotting the relative peak area (RPA) versus the concentration. Coefficient of determination (R^2) was calculated and used for assessing linearity. The mixed-standard solution was diluted with MeOH to obtain 6 concentrations ranging from 5 to 200 µg/mL of each standard marker. IS solution (50 µL) was added to each concentration level (1 mL) prior to subjecting to HPLC analysis.

Precision of the method was evaluated for intra-day and inter-day precisions. CTL extract was analyzed 6 times consecutively for intra-day precision testing. CTL extracts were successively analyzed on 3 different days to evaluate inter-day precision. Percentages of relative standard deviation (%RSD) for both intra-day and inter-day precisions were calculated.

Accuracy was analyzed using the standard addition method. The known amounts of the mixed-standard solutions (25, 50, and 100 µg/mL) were spiked into the CTL extract. Analysis of each spike level was carried out in triplicate, and the percentage of recovery was calculated.

LOD and LOQ were estimated based on signal-to-noise ratio for the sample solution. The minimum concentrations of the markers, which showed signal-to-noise ratios at 3:1 and 10:1, were considered as LOD and LOQ, respectively.

Stability of the method was evaluated using %RSD of RPA at different points of time. CTL extract was subjected to HPLC analysis at 0, 100, 200, 300, 400 and 500 min after extract preparation.

2.7 Establishment of HPLC fingerprints for qualitative (Cai *et al.*, 2010) and quantitative analysis of markers

Reference batches of CTL and 8 herbal components were analyzed under the optimized HPLC conditions. The similarity of chromatographic patterns among the reference batches was assessed from Pearson's correlation coefficients using relative retention time (RRT) of all peaks in a chromatogram. Critical parameters for identification and qualitative analysis including the common peaks, RPA of common peaks, strong peaks, and characteristic peaks, were established according to the information obtained from 12 fingerprints.

2.8 Quality assessment of commercial CTL products using the combinative HPLC analysis

Eight commercial CTL products of different brands (P1 to P8) were prepared and analyzed. All the commercial products were analyzed within 3 years from manufacturing date. Qualitative analysis was evaluated by comparing the chromatogram of the test sample to the CTL reference fingerprints. The critical parameters including common peak, characteristic peak, RPA of common peaks, strong peaks, and degrees of similarity, were evaluated according to the acceptance criteria described in Table 1 (Cai *et al.*, 2010). Quantitative analysis was determined for the contents of markers and was compared with mean quantities of markers in the 12 reference batches.

2.9 Data analysis

One-way ANOVA was used to compare the contents of 4 markers in CTL extract between different extraction solvents. The significance threshold was set at a 95% confidence level. The fingerprint similarity was evaluated by Pearson's correlation analysis carried out using Microsoft® Excel.

3. Results and Discussion

3.1 Characteristics of CTL chromatogram

The developed HPLC conditions successfully separated chemical components of CTL within 100 min, so that the peaks corresponding to standard markers had a resolution greater than 1.2 and other peaks showed a peak to valley ratio greater than 2/3 of that peak (Cai *et al.*, 2010). Borneol was not suited for detection by HPLC-DAD, and ChP (2010) recommended use of gas chromatography for borneol assay. Eurycomanone, α-santalol, artemisinin, apigenin, imperatorin, atractylodin and loureirin A were considered the standard markers to represent quality of *E. longifolia*, *S. album*, *Ar. annua*, *T. crispa*, *An. dahurica*, *At. lancea* and *D. Cochinchinensis*, respectively (Figure 1). These compounds have been researched for their known antipyretic activity (Sindhu, & Arora, 2013) or other recognized pharmacological effects such as anti-inflammatory and anti-malarial effects (Chae, Kim, & Chin, 2016; Fan *et al.*, 2014; Kozioł, & Skalicka-Woźniak, 2016; Mohamed, Vejayan, & Yusoff, 2015; Zhou, Wang, Zhou, Song, & Xie, 2017). Unfortunately, α-santalol was not commercially available and was excluded from the marker list of CTL.

At 254 nm, eurycomanone, loureirin A, imperatorin and atractylodin were detected with satisfactory resolution and sensitivity. Detection at 300 nm was specific to atractylodin, while eurycomanone, loureirin A, and imperatorin signals decreased from those at 254 nm (Figure 2). Thus, the quantitative wavelength for analysis of CTL extract via all the marker compounds should be set at 254 nm. Artemisinin was undetectable due to the absence of a specific chromophore. Some studies have solved this problem (Ghafoori *et al.*, 2013), but derivatization might affect other compounds in CTL and was not appropriate for quantitative analysis. Quantitative analysis of artemisinin in CTL should be performed separately. Interestingly, at 300 nm, 2 peaks which correspond to *Ar. annua* were found in CTL extract at retention times of 33.4 and 35.0 min (Figure 2). These peaks might be used for controlling the quality of *Ar. annua* in the preparation.

Table 1. Acceptance criteria of fingerprint parameters

Parameter	Acceptance criteria
Common peaks	Not less than 90% of reference fingerprints present a common peak identified by relative retention time and UV sensor
Characteristic peaks	Must be 100% present in reference fingerprints, as identified by relative retention time and UV sensor
Strong peaks	The total peak area of the strong peaks should exceed 70% of the total peak area in chromatogram
Degrees of similarity	Degree of similarity between test sample and reference fingerprint is calculated based on the common peak pattern. Total degree of similarity will be less than or equal to 1. $Degree\ of\ similarity = \frac{A_I^T}{A_I^F} x100\%$ $Total\ degree\ of\ similarity = \frac{\Sigma \frac{A_I^T}{A_I^F}}{n}$ In these formulae, A_I^T is RPA of each common peak in the chromatogram of the test sample, A_I^F is RPA of each common peak in the reference fingerprint, n is the total number of the common peaks

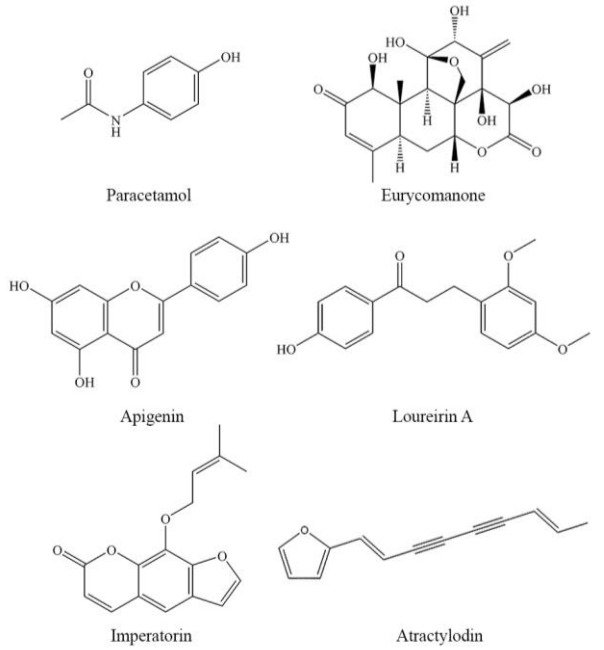

Figure 1. Chemical structures of CTL standard markers

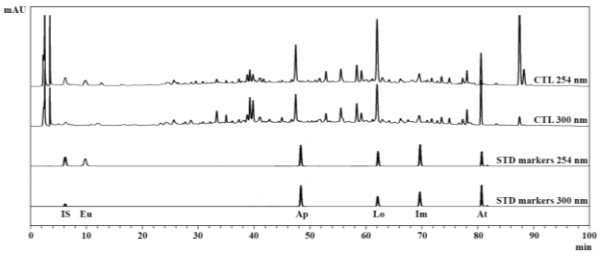

Figure 2. HPLC Chromatogram of CTL detection at UV 254 nm and at UV 300 nm compared with standard markers including eurycomanone (Eu), apigenin (Ap), loureirin A (Lo), imperatorin (Im) and atractylodin (At).

3.2 Optimization of extraction solvents

Extraction solvent is a key factor for extraction efficiency (Li *et al.*, 2010). Water extract was found to give the greatest extraction yield at 17.65 ± 1.84 %w/w, which was 6 times greater than the lowest yield for hexane extract (2.99 ± 0.21 %w/w). Considering the extraction efficiency of markers, MeOH was the most appropriate solvent for CTL extraction. CTL methanolic extract yielded the highest amounts of 3 markers, but not for eurycomanone (Figure 3). Water was suitable for the extraction of eurycomanone, but it was not appropriate to extract the other markers. In contrast, low polarity solvents were appropriate for the extraction of loureirin A, imperatorin and atractylodin. Eurycomanone content in CTL methanolic extract was rather low. Further optimization of extractive solvents was performed using water in combination with MeOH. Interestingly extraction with 80%v/v MeOH significantly improved eurycomanone content (0.252 ± 0.031 mg/g) when compared to MeOH (0.184 ± 0.016 mg/g) and maintained a high extraction efficiency for the other low polarity markers comparably to the methanolic extract. Addition of water in solvent extraction to 50%v/v

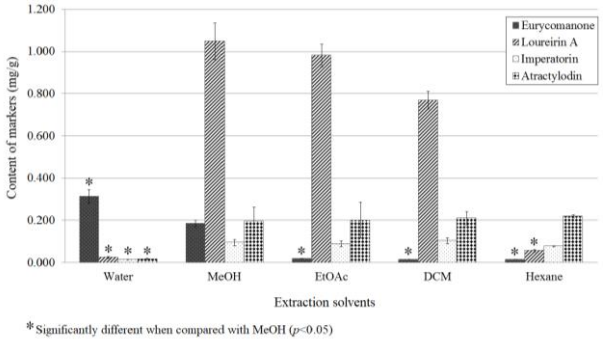

Figure 3. Effects of extraction solvent choice on CTL markers in CTL extracts (n=3)

significantly reduced atractylodin content (0.026 ± 0.005 mg/g) when compared with MeOH (0.196 ± 0.067 mg/g). The results suggest that 80%v/v MeOH is the most appropriate choice for CTL extraction.

3.3 Establishment of CTL chromatographic fingerprints for qualitative and quantitative analysis

The overall chromatographic patterns of CTL1 to CTL12 were generally consistent with some slight differences in number of peaks and peak areas due to the variations in raw materials. The fingerprint similarity was evaluated by Pearson's correlation analysis, which has been widely used for chromatogram similarity analysis (Goodarzi *et al.*, 2013; Tistaert *et al.*, 2011). The correlation analysis used relative retention times of all peaks in the fingerprint. The results showed a high correlation coefficient exceeding 0.9, implying that the 12 reference batches were not significantly different in their peak patterns.

Based on the number of total peaks, peak area, peak positions and spectra obtained for the 12 reference batches, the CTL reference fingerprints were established (Figure 4). Common peaks label was assigned to each peak that existed in all reference batches. The common peaks that corresponded to standard markers were specified as "characteristic peaks". The number of strong peaks was identified based on peak areas. The first 70% of the total peak area were selected as "strong peaks" that indicate the relative amount of the main components (Cai *et al.*, 2010).

At 254 nm, fingerprints of the 12 reference batches showed 139-159 peaks with at least 26 peaks assigned as common peaks (Figure 4). Peaks No. 1, 15, 18 and 24 were assigned as characteristic peaks that correspond to eurycomanone, loureirin A, imperatorin and atractylodin, respectively. Peaks No. 6, 8, 10, 13, 16, 18, 24, 25 and 26 were selected as strong peaks. At 300 nm, there were 27 common peaks. The numbers of common peaks were assigned the same as they were assigned at 254 nm. The numbers of the absent peaks were skipped. Symbols α, β or γ were consequently used between two consecutive peak Nos. which appeared only at 300 nm. Peaks No. 5, 6, 8, 10^{α}, 10^{β}, 11^{α}, 13, 17 and 24 were selected as strong peaks at 300 nm.

A previous publication reported HPLC fingerprints of CTL aqueous extract, but was unable to identify which compound was derived from any herbal component in the preparation and left the quantities of important compounds

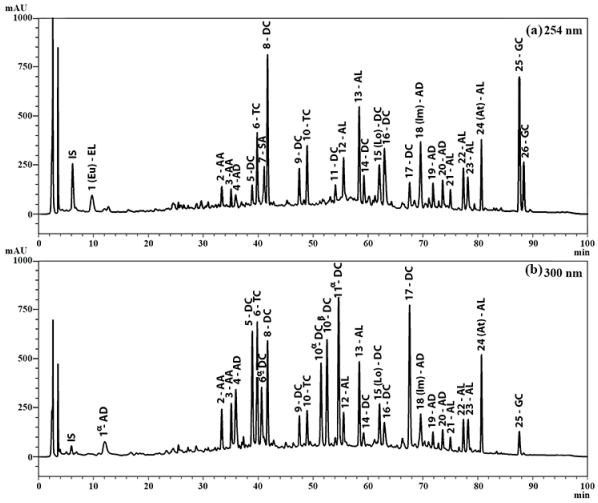

Figure 4. Established CTL chromatographic fingerprints. Common peaks for the herbal components correspond to *E. longifolia* (EL), *S. album* (SA), *Ar. Annua* (AA), *T. crispa* (TC), *An. dahurica* (AD), *G. chinense* (GC), *At. lancea* (AL) and *D. cochinchinensis* (DC)

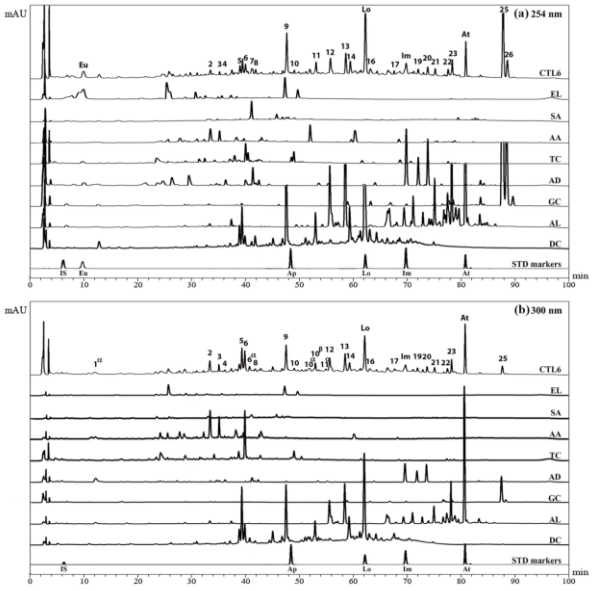

Figure 5. HPLC chromatograms of CTL6 and its individual herbal components

undetermined (Wattanarangsan *et al.*, 2007). In this work, the established fingerprints reflect the quality and authenticity of each herbal component in the preparation. All the common peaks were completely represented in all herbal components. *D. cochinchinensis*, *T. crispa*, *G. chinense*, *At. lancea* and *An. dahurica* are the major contributors of the fingerprints for both common peaks and strong peaks. Peaks No. 1, 2-3, and 7 revealed the constituents *E. longifolia*, *Ar. annua* and *S. album*, respectively (Figure 5).

3.4 Validation of analytical method

3.4.1 Validation of the qualitative analytical method

Intra-day and inter-day precision and stability are crucial parameters, which must be validated for a qualitative analytical method. Validation of intra-day precision showed that all the common peaks gave low %RSD in the range from 0.16 to 1.68%. The inter-day precision of all common peaks was in satisfactory range with %RSD in the range from 0.1 to 5.9%. The retention time of each common peak was within ± 0.06 min of the average retention time. These results indicate that the developed method exhibited high precision. The stability of the test sample was evaluated. It was found that CTL extract exhibited high stability within 8 h. All the common peaks were stable during an analytical time of 8 h, with %RSD from 0.30 to 1.68%.

3.4.2 Validation of quantitative analytical method

The homogeneity of the UV absorption spectrum was used for specificity validation. All the markers showed high specificity with the purity index of each peak higher than 0.9. Linearity validation was performed by constructing the calibration curves of 4 standard markers. The calibration curves exhibited good linearity ($R^2 > 0.998$) in a concentration range of 5-200 µg/mL. The LOQ for all the markers was sufficiently low for their quantification in the CTL extract. Loureirin A, imperatorin and atractylodin showed satisfactory sensitivity with LOQ of 9.89, 5.19 and 4.64 µg/mL, respectively. Eurycomanone showed a poor sensitivity with LOQ of 26.52 µg/mL, but this was good enough to quantify this compound in CTL. The method exhibited high precision. The %RSD values of 4 markers were all less than 0.74% for intra-day precision and 1.55% for inter-day precision. These results indicate that the precision of the system was excellent. All recovery ranges were from 93.24 to 102.91%. These data show that the developed method is suitable and provided reliable test results (Table 2).

3.5 Quality assessment of commercial CTL products using the combinative HPLC analysis

Most commercial CTL products showed similar patterns and complied with the reference fingerprints (Figure 6). The critical parameters including common peaks, characteristic peaks, and strong peaks P1-P5, P7 and P8 were complied to with the acceptance criteria of CTL reference fingerprints. The degrees of similarity ranged from 0.62 to 0.90 revealing inconsistency in the raw material quality. Using substitute plants or misidentification of plant material along with low content of chemical constituents was found in the commercial CTL products. Since *S. album* is an endangered species, Thai national drug list suggests using *Tarenna hoaensis* as a replacement. The signal of peak No. 7 which corresponds to *S. album* was absent in P4 and P7 but some signals that correspond to *T. hoaensis* appeared at 28 min for both formulations (Figure 6). The misidentification of *Ar. annua* was found in all commercial products. It was found that *Ar. vulgaris* was used instead of *Ar. annua*. Characteristic peaks No. 2 and 3 were used for identifying *Ar. annua*. The *Ar. vulgaris* extract showed different characteristic peaks, and some peaks overlapped with eurycomanone at 10 min. This HPLC fingerprint is useful for raw material identification and determination of adulteration in the CTL preparation.

Table 2. Validation parameters for the simultaneous quantification of 4 markers in CTL

Parameter	Markers			
	Eurycomanone	Loureirin A	Imperatorin	Atractylodin
Linearity				
Concentration range (µg/mL)	5 - 200	5 - 200	5 - 200	5 - 200
R^2	0.9983	0.9983	0.9983	0.9986
Slope	0.0071	0.0118	0.0272	0.0187
Linear coefficient	- 0.0202	- 0.0276	- 0.0649	- 0.0495
Precision (%RSD of RPA)				
Repeatability; n=6	0.74	0.37	0.40	0.67
Intermediate precision; 3 days	1.15	0.31	1.55	0.10
Accuracy (%recovery, RSD)				
Low level; n=3	93.24 (1.22)	100.46 (1.80)	98.87 (1.17)	98.60 (1.08)
Medium level; n=3	93.73 (1.61)	100.73 (0.86)	100.95 (0.54)	102.91 (0.84)
High level; n=3	102.77 (0.25)	102.58 (1.04)	100.32 (1.18)	101.14 (1.07)
Limit of Detection (µg/mL)	8.97	3.39	1.70	1.60
Limit of Quantitation (µg/mL)	26.52	9.89	5.19	4.64

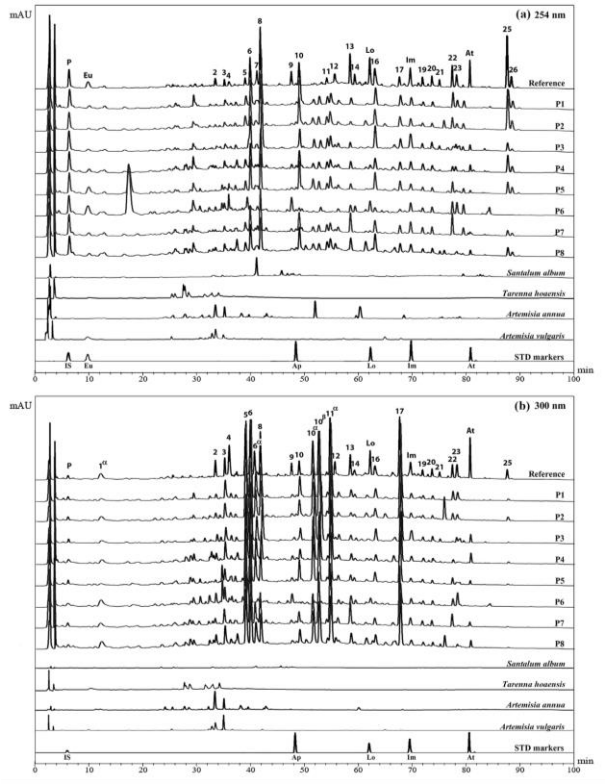

Figure 6. HPLC chromatograms of commercial CTL products of different brands: (a) detection at UV 254 nm, and (b) detection at UV 300 nm

However, this method was unable to analyze eurycomanone if *Ar. vulgaris* was used instead of *Ar. annua* in the preparation. Thin layer chromatography or HPLC-MS/MS might be able to solve the specificity issue.

The contents of loureirin A, imperatorin and atractylodin in all the commercial products were in the ranges from 0.014 to 0.265 mg/g, 0.074 to 0.161 mg/g, and 0.017 to 0.081 mg/g, respectively (Table 3). The marker content in commercial products was relatively low when compared to the reference batches, and this may affect the curative effects. Eurycomanone content could not be determined due to the peak overlapping with *Ar. vulgaris*. The inconsistency observed in the chemical contents in CTL was due to large variations in the herbal materials. Selection of high-quality raw material sources is a key to achieving a reliable curative effect. Thus, auditing vendors or plant material sources is necessary for a manufacturer to ensure the therapeutic effects of the products and compliance with good manufacturing practices.

4. Conclusions

Quantitative analysis of markers and qualitative analysis of fingerprints are powerful techniques that can provide information for quality assessment of herbal medicines. The qualitative approach using chromatographic fingerprints plays a major role in the identification of markers and potential unknown compounds present in the complex matrix.

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Table 3. Contents of markers in 12 reference batches (CTL1-CTL12) and in 8 commercial products (P1-P8)

Sample	Content of markers, mg/g			
	Eurycomanone	Loureirin A	Imperatorin	Atractylodin
CTL[a]	0.284 ± 0.161	0.466 ± 0.470	0.165 ± 0.068	0.228 ± 0.107
P1[b]	-	0.016 ± 0.001	0.094 ± 0.004	0.017 ± 0.001
P2[b]	-	0.014 ± 0.000	0.089 ± 0.003	0.018 ± 0.000
P3[b]	-	0.017 ± 0.001	0.161 ± 0.007	0.065 ± 0.001
P4[b]	-	0.102 ± 0.006	0.091 ± 0.003	0.081 ± 0.003
P5[b]	-	0.017 ± 0.000	0.074 ± 0.003	0.039 ± 0.001
P6[b]	-	0.265 ± 0.009	0.104 ± 0.005	0.017 ± 0.000
P7[b]	-	0.058 ± 0.002	0.122 ± 0.004	0.037 ± 0.001
P8[b]	-	0.018 ± 0.001	0.110 ± 0.005	0.065 ± 0.003

[a] Expressed as mean ± SD in 12 reference batches (CTL1-CTL12).
[b] Expressed as mean ± SD in each commercial product (P1-P8).

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