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Original Article

Simultaneous HPTLC quantification of three caffeoylquinic acids in *Pluchea indica* leaves and their commercial products in Thailand

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ABSTRACT

In Thai traditional medicine, *Pluchea indica* (L.) Less., Asteraceae, leaf has been widely used for the treatment of diabetes mellitus, tumors, hypertension, cystitis, and wounds. *P. indica* herbal tea is commercially available in Thailand as a health-promoting drink. The study was conducted to develop and validate a high-performance thin-layer chromatography (HPTLC) method for the quantitative analysis of chlorogenic acid, 3,4-*O*-dicaffeoylquinic acid, and 3,5-*O*-dicaffeoylquinic acid in *P. indica* leaf extract and their commercial products in Thailand. The method was validated according to ICH guidelines. The proposed HPTLC method showed acceptable validation parameters. The content of chlorogenic acid, 3,4-*O*-dicaffeoylquinic acid, and 3,5-*O*-dicaffeoylquinic acid in *P. indica* leaves from seven different provinces in Thailand was in the range of not detectable -1.94 ± 0.02%w/w, 0.71 ± 0.01–1.89 ± 0.05%w/w, and 1.00 ± 0.01–2.18 ± 0.03%w/w, respectively, while in the commercial products, it was in the range of 0.59 ± 0.03–2.17 ± 0.05%w/w, 0.53 ± 0.04–3.77 ± 0.03%w/w, and 0.88 ± 0.05–4.72 ± 0.10%w/w, respectively. The results indicated that plantation of *P. indica* in coastal saline land would be beneficial as it would increase the concentration of its active compounds and improve its medicinal quality. The developed high-performance thin-layer chromatography could be used as a rapid, reliable, less demanding, and cost-effective analytical method.

Introduction

Pluchea indica (L.) Less., belonging to Asteraceae family, is a perennial shrub species found in Australia, and many Asian countries such as India, Indonesia, Malaysia, Taiwan, and Thailand. It can reach up to 2 m in height, and it naturally grows in wet saline habitats (eFloras, 2008). *P. indica* leaves have a natural sweet taste and a biting flavor and normally consumed as a side dish or herbal tea (Office of Mangrove Resources Conservation, 2009). This herbal tea has a pleasant flavor, is light to dark green-brown color, and also used in traditional medicine owing to its diuretic, antipyretic, anti-inflammatory, antidiabetic, and antioxidant properties (Pramanik et al., 2006; Buapool et al., 2013; Widyawati et al., 2014; Vongsak et al., 2018). In addition, previous phytochemical investigations showed that the leaf extracts contain caffeoylquinic derivatives such as chlorogenic acid (CGA), 3,4-*O*-dicaffeoylquinic acid (3,4 diCQA), 3,5-*O*-dicaffeoylquinic acid (3,5 diCQA), quercetin, kaempferol, myricetin, monoterpenes, and sesquiterpenoids (Andarwulan et al., 2010; Widyawati et al., 2013; Arsiningtyas et al., 2014; Kongkiatpaiboon et al., 2018). CGA has antioxidant, antibacterial, anti-inflammatory, and hypoglycemic properties (Yu et al., 2018). diCQAs are potent alpha glucosidase inhibitors and prevent virus replication in tissue culture (McDougall et al., 1998; Arsiningtyas et al., 2014). Therefore, research on these compounds is gaining importance.

Several analytical methods have been reported for the investigation of the phytochemical compounds in *P. indica* extract such as GC and GC–MS, HPLC–MS/MS, and HPLC–DAD (Le et al., 2000; Shukri et al., 2011; Kongkiatpaiboon et al., 2018). Each method has its own limitations. For instance, GC is used for the examination of volatile compounds. Volatile compounds are vaporized at the inlet temperature and separated later in the gas phase. This separation makes GC perfect for the investigation of volatile complex compounds but this technique is not useful for non-volatile constituents (Heshka and Hager, 2015). HPLC analysis involves the transfer of compounds from one mobile phase to another to elute at different times; however, this complex functionality is associated with miscibility issues and high back pressure (Petruczynik et al., 2008). High-performance thin-layer chromatography (HPTLC)

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Box 1: Different brands of *Pluchea indica* analyzed in this study.

Brand	Content
A	Loose *Pluchea indica* leaf tea
B	Loose *Pluchea indica* leaf tea
C	*Pluchea indica* herbal tea. Each sachet contained 1 g of *P. indica* leaves
D	*Pluchea indica* herbal tea. Each sachet contained 1 g of *P. indica* leaves
E	*Pluchea indica* herbal tea. Each sachet contained 1.5 g of *P. indica* leaves
F	*Pluchea indica* capsule. Each capsule contained 250 mg of *P. indica* leaf, root, stem, and flower powder
G	*Pluchea indica* capsule. Each capsule contained 250 mg of *P. indica* leaf powder
H	*Pluchea indica* capsule. Each capsule contained 300 mg of *P. indica* leaf powder

offers lesser area for separation of compounds and has lesser plate efficiency than HPLC and GC but it remains a valuable technique for the quality control analysis of herbal medicines as it is simple, cost-effective, and less demanding. It has been commercially utilized in fingerprint analysis to characterize and quantify numerous commercial herbal formulations and natural products at nanogram levels (Kharat et al., 2017).

Thus, the aim of the study was to develop and validate an HPTLC analytical method for the quality control of *P. indica* leaf extract using CGA, 3,4 diCQA, and 3,5 diCQA as quality markers. Several samples obtained from natural habitats and commercial products were analyzed.

Materials and methods

Chemicals and reagents

Standard CGA, 3,4 diCQA, and 3,5 diCQA, (purity >98%) were purchased from Chengdu Biopurify Phytochemicals Ltd., China. All other reagents used were of analytical grade.

Plant material

Mature leaves of *Pluchea indica* (L.) Less., Asteraceae, were collected from the following seven different provinces in Thailand: (1) Chanthaburi; (2) Chonburi; (3) Petchaburi; (4) Samut Songkhram; (5) Uttaradit; (6) Nakhon Ratchasima; (7) Songkhla; from July to August 2016. The specimens (voucher number PB16001–PB16007) were identified using the identification key provided in Flora of Thailand and deposited at Faculty of Pharmaceutical Sciences, Burapha University, Chonburi, Thailand. The leaves were washed with tap water and dried in a hot air oven (Memmert, Germany) at 50 °C until a constant weight was achieved. The dried leaves were ground to pass through a 0.5-mm sieve and kept at −20 °C for further studies.

Commercial products including two samples of *P. indica* loose leaves tea, three samples of *P. indica* herbal tea, and three samples of *P. indica* capsules were purchased from local herbal drug stores in Bangkok as shown in Box 1.

Sample extraction

From our previous study (Kongkiatpaiboon et al., 2018), ultrasound extraction with 50% ethanol yielded the highest concentration of caffeoylquinic acid derivatives in the *P. indica* leaf extract. Briefly, the powdered leaves were extracted with 50% ethanol (1:20, w/v) using an ultrasonic bath. The extraction was performed in triplicate. The pooled extract was filtered through Whatman no. 1 paper. The filtrate was dried under reduced pressure at 50 °C using a rotary evaporator. The crude extract was kept in a tight container protected from light at 0 °C.

For commercial products, each sample was ground to pass through a 0.5-mm sieve, then extracted with 50% ethanol as described above.

Preparation of sample solution

Each sample was prepared by accurately weighing (0.1 g) *P. indica* leaf extract, dissolved in methanol and adjusted to 10 ml in a volumetric flask. After complete dissolution by sonicating for 30 min, each solution was filtered through a 0.45-μm nylon membrane filter before being applied to the HPTLC plate (2 μl/band; $n = 3$).

Preparation of standard solution

CGA, 3,4 diCQA, and 3,5 diCQA standards were accurately weighed and dissolved in methanol in a volumetric flask for the preparation of stock solutions (1 mg/ml). Working standard solutions of CGA, 3,4 diCQA, and 3,5 diCQA were prepared to obtain a final concentration of 100 μg/ml.

Instrument and chromatographic condition

HPTLC was performed on an aluminum sheet of silica gel 60 F254 (20 cm × 10 cm, Cat. No. 1.05548.0001, Merck). Sample and standard solutions were applied to the plate as 7-mm bands with a Linomat V automatic sample spotter (Camag, Switzerland) under nitrogen flow, positioned at 10 mm from the bottom of the plate. The constant application rate was 150 nl/s. The mobile phase consisted of ethyl acetate:water:formic acid:toluene (20:2:2:1, v/v). The plate was developed to a distance of 8 cm in a Camag twin trough chamber. Densitometric scanning was performed using a TLC scanner 4 (Camag, Switzerland) using the WinCATS software. The wavelength of detection was 326 nm. The dimension of the slit was 6.0 × 0.45 mm, and the scanning speed was 10 mm/s.

Method validation

The method was validated by evaluating linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness according to the ICH guidelines (ICH, 1996/2005).

Linearity

From working standard solutions of CGA, 3,4 diCQA, and 3,5 diCQA (100 μg/ml), 1–4 μl of each solution was applied to the HPTLC plate, corresponding to concentrations of 100–400 ng/band. Calibration (linearity) curves were obtained by plotting the peak area versus the concentration of each standard.

Precision

The precision of the analytical method for each compound was determined by analyzing 200, 300, and 400 ng/band of CGA, 3,4 diCQA, and 3,5 diCQA standard solutions after applying to the HPTLC plate three times on the same day for intraday precision and on three different days for interday precision. The precision was expressed as percent relative standard deviation (% RSD).

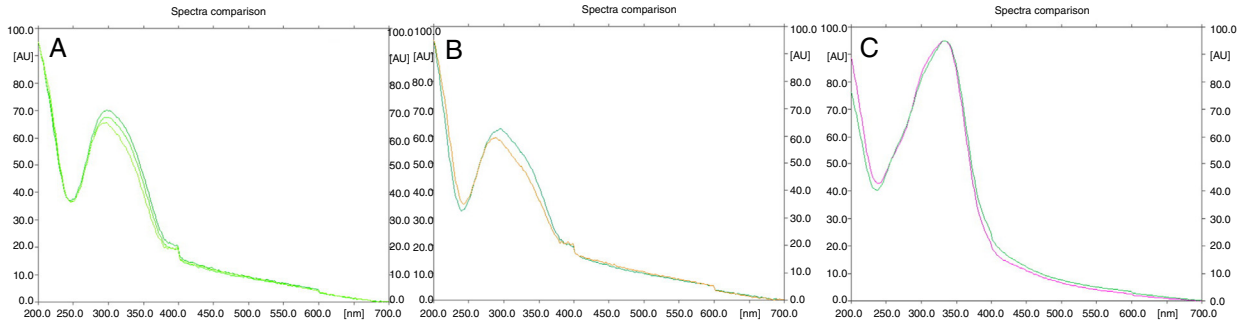

Fig. 1. Overlay UV spectra scanning from 200 to 700 nm by HPTLC method. (A) CGA reference standard and sample; (B) 3,4 diCQA reference standard and sample; (C) 3,5 diCQA reference standard and sample.

Accuracy

The accuracy of the method for each compound was evaluated by determining recovery. The recovery of CGA, 3,4 diCQA, and 3,5 diCQA was performed on a sample spiked with three concentrations of standards (50%, 100%, and 150% of the determined content of *P. indica* leaves) ($n = 3$).

LOD and LOQ

LOD and LOQ were determined based on the standard deviation (SD) of the response and the slope (S) of each calibration curve of CGA, 3,4 diCQA, and 3,5 diCQA according to the formula: LOD = 3.3(SD/S) and LOQ = 10(SD/S).

Robustness

The robustness of the method was determined by changing some chromatographic conditions at a level of 300 ng/band of CGA, 3,4 diCQA, and 3,5 diCQA. The solvent system composition was slightly changed (±5%) as 20:2:2:1, 18.75:2:2:1, 21.25:2:2:1, v/v, of ethyl acetate:water:formic acid:toluene. The time between spotting CGA, 3,4 diCQA, and 3,5 diCQA onto the HPTLC plate to development was varied in the range of 5, 15, and 30 min. The time after development to densitometric scanning was varied in the range of 5, 15, and 30 min. The %RSD of the peak areas of the CGA, 3,4 diCQA, and 3,5 diCQA reference standards were calculated for all robustness variations.

Results and discussion

The mobile phase consisted of ethyl acetate:formic acid:water:toluene (20:2:2:1, v/v) gave the best peak resolution of CGA, 3,4 diCQA, and 3,5 diCQA. The specificity of the bands of CGA ($Rf = 0.34$), 3,4 diCQA ($Rf = 0.63$), and 3,5 diCQA ($Rf = 0.79$) in *P. indica* leaf extract was confirmed by overlaying the absorption spectra of the samples with those of CGA, 3,4 diCQA, and 3,5 diCQA reference standards (Fig. 1). The specificity of the analyzed peaks was checked at three different peak levels i.e., start, apex, and end positions of the peaks corresponding to CGA, 3,4 diCQA, and 3,5 diCQA.

The proposed HPTLC method showed acceptable validation parameters (Table 1). The calibration curve of CGA, 3,4 diCQA, and 3,5 diCQA was linear over the range of 100–400 ng/band. The correlation coefficient value was ≥0.995, confirming the linearity of the method. The % RSD value of intra- and interday precision was <5%. The LOD of CGA, 3,4 diCQA, and 3,5 diCQA was found to be 9.92, 17.58, and 6.68 ng/band, while the LOQ of CGA, 3,4 diCQA, and 3,5 diCQA was found to be 30.05, 53.27, and 20.24 ng/band, respectively. The average recovery of CGA, 3,4 diCQA, and 3,5 diCQA was 99.04 ± 5.21, 97.51 ± 4.14, and 99.64 ± 9.65%, respectively. The validation parameters indicated both good precision and accuracy of the analytical method. For robustness studies, %RSD was found to be less than 5% for all variations.

The content of CGA, 3,4 diCQA, and 3,5 diCQA in *P. indica* leaves from seven different provinces was in the range of not detected −1.94 ± 0.02%w/w, 0.71 ± 0.01–1.89 ± 0.05%w/w, and 1.00 ± 0.01–2.18 ± 0.03%w/w, respectively (Table 2). The HPTLC chromatograms of reference standards, *P. indica* leaf extract, and its commercial products are shown in Fig. 2.

Pluchea indica leaves from the Nakhon Ratchasima province (Northeastern region) contained the lowest amount of three major compounds. It might be because of the poor quality of soil in the northeastern mountains, which are generally less fertile, and because temperatures are considerably warmer here than in the other parts of the country (Choenkwan et al., 2014). *P. indica* commonly grows in saline habitats such as brackish marshes and mangroves. Numerous studies reported that NaCl stress can induce

Table 1
Validation parameters by the proposed HPTLC method.

Parameter	CGA	3,4 diCQA	3,5 diCQA
Range of linearity	100–400 ng/band	100–400 ng/band	100–400 ng/band
Regression equation ($n = 4$)	$Y = 111.28 + 8.79$ X	$Y = -444.19 + 9.37X$	$Y = -379.78 + 21.05X$
Correlation coefficient (r^2)	0.99501 ± 0.0028	0.99863 ± 0.0010	0.99618 ± 0.0039
% Recovery	99.04 ± 5.21%	97.51 ± 4.14%	99.64 ± 9.65%
% RSD intraday precision	0.20–2.40%	1.21–2.68%	1.16–1.91%
% RSD interday precision	0.66–2.70%	2.16–3.05%	1.20–2.53%
Limit of detection (LOD)	9.92 ng/band	17.58 ng/band	6.68 ng/band
Limit of quantitation (LOQ)	30.05 ng/band	53.27 ng/band	20.24 ng/band
Robustness			
Time from application to development	1.60%	1.65%	0.96%
Time from development to scanning	3.86%	0.97%	1.72%
Mobile phase change composition	1.68%	0.61%	3.86%

X = concentration of CGA, 3,4 diCQA, and 3,5 diCQA in ng/μl; Y = peak area; CGA, chlorogenic acid; 3,4 diCQA, 3,4-*O*-dicaffeoylquinic acid; 3,5 diCQA, 3,5-*O*-dicaffeoylquinic acid.

Table 2
Content of CGA, 3,4 diCQA, and 3,5 diCQA in *Pluchea indica* leaf extract and its commercial products determined by the validated HPTLC densitometric method.

Sample	Content of major compound[a] (%w/w)		
	CGA	3,4 diCQA	3,5 diCQA
***Pluchea indica* leaves (region/location)**			
Eastern			
Chanthaburi	1.06 ± 0.03	1.03 ± 0.04	1.02 ± 0.04
Chonburi	1.94 ± 0.02	1.77 ± 0.04	2.00 ± 0.01
Western			
Petchaburi	Not detected	1.13 ± 0.01	1.39 ± 0.04
Central			
Samuth Songkhram	0.84 ± 0.01	0.91 ± 0.02	1.00 ± 0.01
Northern			
Uttaradit	1.12 ± 0.02	1.03 ± 0.02	1.00 ± 0.02
North eastern			
Nakhon Ratchasima	0.80 ± 0.01	0.71 ± 0.01	1.08 ± 0.02
Southern			
Songkhla	0.71 ± 0.04	1.89 ± 0.05	2.18 ± 0.03
Commercial product			
Loose leaf tea			
Brand A	1.77 ± 0.03	2.03 ± 0.05	1.04 ± 0.04
Brand B	1.35 ± 0.07	1.85 ± 0.12	2.12 ± 0.11
Herbal tea			
Brand C	2.17 ± 0.05	1.24 ± 0.05	1.93 ± 0.05
Brand D	0.59 ± 0.03	0.53 ± 0.04	0.96 ± 0.06
Brand E	1.00 ± 0.08	0.55 ± 0.02	0.88 ± 0.05
Capsule			
Brand F	1.37 ± 0.02	2.81 ± 0.04	3.98 ± 0.14
Brand G	0.84 ± 0.04	1.87 ± 0.11	2.37 ± 0.15
Brand H	1.91 ± 0.02	3.77 ± 0.03	4.72 ± 0.10

[a] Expressed as mean ± SD (n = 3); CGA, chlorogenic acid; 3,4 diCQA, 3,4-*O*-dicaffeoylquinic acid; 3,5 diCQA, 3,5-*O*-dicaffeoylquinic acid.

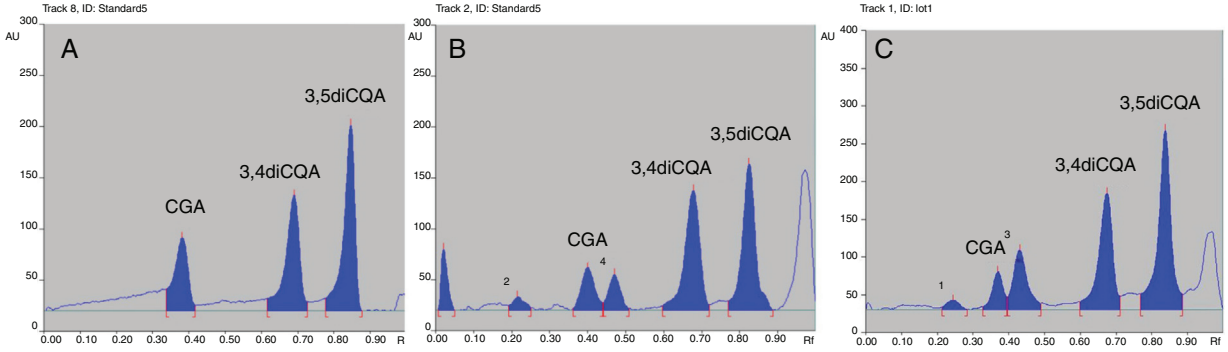

Fig. 2. HPTLC chromatograms. (A) CGA, 3,4diCQA and 3,5 diCQA standard; (B) *Pluchea indica* leaves from Uttaradit province; (C) Commercial product (Brand B).

the accumulation of phenolic compounds in plant tissues. Yan et al. (2016) reported that the concentration of CGA in honeysuckle leaves was higher in saline than in non-saline fields (Yan et al., 2016). Borgognone et al. (2014) also reported that salt stress (NaCl and KCl) could increase total phenolic, total flavonoids, and antioxidant activity in leaves of artichoke and cardoon (Borgognone et al., 2014). In agreement with our result, the content of CGA, 3,4 diCQA, and 3,5 diCQA in *P. indica* leaf extract from coastal provinces (Songkhla, Chanthaburi, and Chonburi) was higher than that in the extract from other regions. Therefore, plantation of *P. indica* in coastal saline land would be beneficial as it would increase the concentration of its active compounds and improve its medicinal quality.

For *P. indica* commercial products, the content of CGA, 3,4 diCQA, and 3,5 diCQA was in the range of 0.59 ± 0.03–2.17 ± 0.05%w/w, 0.53 ± 0.04–3.77 ± 0.03%w/w, and 0.88 ± 0.05–4.72 ± 0.10%w/w, respectively (Table 1). Drying reduces moisture content, thereby inhibiting enzymatic degradation and limiting bacterial growth in herbal tea products. This essential step in tea processing influences the appearance and antioxidant activity of tea, which affect the its commercial value (Chong and Lim, 2012). Drying significantly affects the content of caffeoylquinic acid derivatives including CGA, 4,5 diCQA, 3,5 diCQA, 3,4 diCQA, and 3,4,5 triCQA in sweet potato leaves. Hot air drying at 70 °C and 100 °C significantly decreases the amount of caffeoylquinic acid derivatives and their antioxidant activities. Among the different drying methods, freeze drying and cool air drying at 30 °C were found to retain higher amounts of caffeoylquinic acid derivatives (Jeng et al., 2015). Similarly, oven drying under higher temperatures (70 °C and 100 °C) significantly decreases total phenolic content and antioxidant activity compared with heat treatment at 40 °C and 50 °C for *Vitex negundo* L. tea (Rabeta and Vithyia, 2013). Katsube et al. (2009) also reported that an air drying at 70 °C significantly decreased DPPH radical scavenging activity and the content of polyphenolic compounds of mulberry leaf (Katsube et al., 2009). The decrease in antioxidant activity during heat treatment could be due to the initial enzymatic degradation and thermal degradation of antioxidant compounds (Cho et al., 2017). Moreover, caffeoylquinic acids are degraded by high temperature and light irradiation (Xue et al., 2016). In accordance with our results, the *P. indica* loose leaf tea and herbal tea formulations showed lower content of the three major compounds than the capsule formulation. It might due to exposure to high

temperatures (pan firing/steaming and oven drying) during the manufacturing process of loose leaf tea. Thus, exposure to high drying temperatures should be avoided during the manufacturing process of *P. indica* products and the products must be stored in light-proof containers.

HPTLC was developed for quantification of CGA, 3,4 diCQA, and 3,5 diCQA in *P. indica* leaf extract and its commercial products. The developed method showed acceptable validation parameters according to ICH guidelines. HPTLC is a simple, rapid, and cost-effective analytical technique. It is one of the most widely applied method for the analysis of herbal extracts, which have a complex mixture of many chemical constituents. It is used for screening for adulterations, quantitative analysis of major compounds, and testing of stability. In conclusion, this developed method can be used as an alternative analytical method in the routine quality control of commercial products of *P. indica* leaves.

Authors' contributions

SC contribution included HPTLC analysis, designing, analyzing the results, and preparing the paper. BV contribution included collecting samples, designing, analyzing the results, and preparing the paper. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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