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Original Article

Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves from different provenances in Thailand

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ABSTRACT

Pluchea indica (L.) Less., Asteraceae, is a medicinal plant which contains a high amount of phenolic compounds such as caffeoylquinic acid derivatives. The leaves have been traditionally used as a nerve tonic and extensively as herbal tea. This study aimed to develop and validate an HPLC method to quantitatively analyze six caffeoylquinic acid derivatives, *viz.* 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O* caffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, and 4,5-*O*-dicaffeoylquinic acid in *P. indica* leaf extract. HPLC was carried out in a Hypersil BDS C_{18}-column eluted with 0.5% acetic acid in water and methanol using gradient elution with a flow rate of 1 ml/min and detection at 326 nm. The method validation was performed to assure its linearity, precision, accuracy and limits of detection and quantitation. Several extraction techniques including maceration, decoction, digestion, Soxhlet extraction, and ultrasound extraction, were used to extract active constituents. The ultrasound extraction with 50% ethanol yielded the highest concentration of these caffeoylquinic acid derivatives in the *P. indica* leaf extract. Our developed HPLC method is simple and reliable for a routine analysis of the six caffeoylquinic acids in *P. indica* leaves and could potentially be applied to be used in commercial herbal products.

Introduction

Pluchea indica (L.) Less., Asteraceae, is an evergreen shrub, commonly known as Indian camphorweed, Indian fleabane and Indian pluchea. It is widely distributed in India, Southern China, Southeast Asia, Australia and the Pacific Islands. It can be found abundantly in brackish marshes, mangrove forest and other saline habitats (eFloras, 2008). Young leaves and shoots are edible and consumed as salad or side-dish to rice. *P. indica* herbal tea has been commercially available in Thailand as a health-promoting drink (Office of Mangrove Resources Conservation, 2009). In Thai traditional medicine, leaves are used as a nerve tonic and for the treatment of inflammation. Whole plants are used for treating hemorrhoids, constipation, aphthous ulcer and gallstone (Srisook et al., 2012; Neamsuvan and Ruangrit, 2017). *Pluchea indica* leaves were found to possess various biological activities including antioxidant (Widyawati et al., 2014), anti-inflammatory (Buapool et al., 2013), hypoglycemic and antihyperglycemic activities (Pramanik et al., 2006).

Previous phytochemical studies demonstrated that leaves of *P. indica* contain caffeoylquinic derivatives (Arsiningtyas et al., 2014), flavonol aglycones (quercetin, kaempferol, myricetin) (Andarwulan et al., 2010), and terpenoid (10*S*,11*S*-himachala-3(12)-4-diene) (Widyawati et al., 2013). An HPLC-PDA-MS study revealed that caffeoylquinic derivatives such as 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid are major compounds of *P. indica* leaves (Shukri et al., 2011). These derivatives also have been shown to possess antioxidant, anti-inflammatory activity, inhibiting HMG-CoA reductase and alpha-glucosidase enzyme (Xu et al., 2012; Vongsak et al., 2013; Chen et al., 2014; Arantes et al., 2016; Motaal et al., 2016). Thus, the six caffeoylquinic derivatives which are the major active compounds could be used as marker compounds for the quality assessment of *P. indica* leaves extract.

Several analytical methods including HPLC (Andarwulan et al., 2010), HPLC-PDA-MS^2 (Shukri et al., 2011), GC and GC–MS (Le et al., 2000) have been reported for the quantification of some

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phytochemical compounds in *P. indica* leaves. However, there has been no previous report on the simultaneous determination of six caffeoylquinic derivatives *viz.* 3-*O*-caffeoylquinic acid (3-CQ), 4-*O*-caffeoylquinic acid (4-CQ), 5-*O*-caffeoylquinic acid (5-CQ), 3,4-*O*-dicaffeoylquinic acid (3,4-CQ), 3,5-*O*-dicaffeoylquinic acid (3,5-CQ), and 4,5-*O*-dicaffeoylquinic acid (4,5-CQ). Therefore, this study was conducted to identify the appropriate extraction method and perform a quantitative HPLC validation of the six caffeoylquinic acids in the *P. indica* leaves extracts collected from various regions in Thailand.

Materials and methods

Chemicals and reagents

HPLC grade methanol was purchased from Labscan (Thailand). Deionized water was purified by Ultra Clear system (Siemen Water Technologies Corp., USA). Glacial acetic acid was purchased from Labscan (Thailand) while all reagents were of analytical grade. 3-CQ, 4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ and 4,5-CQ were purchased from Chengdu Biopurify Phytochemicals Ltd., China.

Plant materials

The mature leaves of *Pluchea indica* (L.) Less., Asteraceae, were collected from fourteen different provinces in Thailand as follows: (1) Laplae District, Uttaradit Province; (2) Muang District Nakhon Ratchasima; (3) Muang District, Nonthaburi; (4) Muang District, Samut Sakhon; (5) Muang District, Samut Songkhram; (6) Tayang District, Petchaburi; (7) Pranburi District, Prachuap Khiri Khan; (8) Paktor District, Ratchaburi; (9) Muang District, Chonburi; (10) Leam sing District, Chanthaburi; (11) Klaeng District, Rayong; (12) Muang District, Phuket; (13) Hadyai District, Songkhla; (14) Phrasaeng District, Surat Thani in July to August, 2016. The specimens, (voucher numbers JAM16001-JAM16014, respectively), 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 cleaned by washing with tap water and a portion was dried in a hot air oven at 50 °C for 24 h. The dried leaves were ground to pass through a 0.5 mm sieve, kept in sealed containers and protected from light until used. For the assessment of a suitable extraction method, the sample from Chanthaburi Province was utilized. The method that provided the highest amount of marker constituents was selected for the sample extraction.

Sample extraction

Maceration extraction

For maceration extraction, the dried powdered leaves were placed in an Erlenmeyer flask, with 50% ethanol (1:20, w/v) at room temperature (28 ± 2 °C) for 72 h with occasional shaking. The plant residue was re-extracted again by the same method.

Decoction extraction

The dried leaf powder was boiled with distilled water (1:20, w/v) at 80 ± 5 °C for 15 min and then filtered. Each extraction method was carried out three times. The marc was re-extracted twice more.

Digestion extraction

The dried powdered leaves were separately digested with 50% ethanol and distilled water (1:20, w/v) for 6 h at 60 ± 5 °C. The extract was filtered and the marc was re-extracted by the same process.

Soxhlet extraction

The dried powder was placed into an extraction thimble and separately extracted with 50% ethanol and distilled water (1:20, w/v) for 6 h until exhaustion.

Ultrasound extraction

The dried powder was placed in an Erlenmeyer flask and extracted separately with 50% ethanol and distilled water (1:20, w/v) using an ultrasonic bath (Bandelin Sonorex Digitec, Type: DT1028H, Germany) with ultrasound 35 kHz at 40 ± 5 °C for 15 min. The marc was re-extracted twice more.

Each extraction method was carried out three times. The combined extracts from each sample were separately filtered through a Whatman No. 1 filter paper. The filtrate was dried under reduced pressure at 50 °C using a rotary evaporator. The crude extract was weighed and kept in a tight container protected from light at 0 °C. Each sample was prepared by accurately weighing *P. indica* extract and dissolving in methanol. To enable a complete dissolution, each sample was sonicated for 30 min. Prior to an injection, each sample was filtered through a 0.22 μm nylon membrane and then analyzed in triplicate.

Stock and working solution of standards

Stock solutions of standard compounds were prepared by accurately weighing and dissolving the compounds in methanol to obtain the final concentration of 1000 μg/ml. Working solution of standard compounds were obtained by diluting the stock standard solutions with methanol to achieve the desired concentrations.

HPLC apparatus and chromatographic conditions

HPLC separation was achieved on an Agilent 1260 Series (Agilent Technologies, USA) equipped with a 1260 Quat pump VL quaternary pump, 1260 ALS autosampler, 1260 TCC column thermostat, and 1260 DAD VL diode array detector. The separation was done in a Hypersil BDS C_{18} column (4.6 × 100 mm i.d., 3.5 μm) with a C_{18} guard column (4 × 10 mm i.d., 3 μm). The mobile phases were (A) 0.5% acetic acid in water and (B) methanol using gradient elution: 10% B in A to 50% B in A for 40 min; 100% B for 10 min. This column was re-equilibrated with 10% B in A for 10 min prior to each analysis and the flow rate was set at 1.0 ml/min with the controlled temperature at 25 °C. DAD detector was set at the wavelength of 326 nm and injection volume was 5 μl for every sample and reference standard.

Method validation

The method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) according to the International Conference on Harmonization guidelines (ICH, 1996/2005).

Linearity

Linearity was determined by using working standard solutions of 3-CQ, 4-CQ, 5-CQ, 3,4-CQ, 3,5-CQ, and 4,5-CQ at concentrations of 150, 75, 37.5, 18.75, 9.38, and 4.69 μg/ml. Each concentration was analyzed in triplicate. The calibration curves were obtained by plotting the peak area versus the concentration of each standard.

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