Revista Brasileira de Farmacognosia 27 (2017) 50–53

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Validated high performance thin layer chromatography method for simultaneous determination of quercetin and gallic acid in *Leea indica*

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ARTICLE INFO

Article history:
Received 14 October 2015
Accepted 24 May 2016
Available online 19 September 2016

Keywords:
Gallic acid
HPTLC
Leea indica
Quercetin
Simultaneous estimation
Validation

ABSTRACT

A sensitive and reliable high performance thin layer chromatography method has been developed for the simultaneous estimation of quercetin and gallic acid in *Leea indica*, Vitaceae. Ethyl acetate extract prepared from hydrolysed aqueous alcoholic extract (70%) was applied on silica gel G 60 F_{254} plate. The plate was developed using toluene-ethyl acetate-formic acid, 5:4:1 (v/v/v) as a mobile phase and detection and quantification were performed by densitometric scanning at 254 nm. The system was found to give well resolved bands for quercetin (R_f 0.63) and gallic acid (R_f 0.45) from other constituents present in the extract of *L. indica*. The correlation coefficient was found to be 0.991 and 0.999 with relative standard deviation, 0.97–1.23% and 0.1–1.13% for quercetin and gallic acid respectively in the developed method. The accuracy of the method was confirmed by conducting recovery studies at different levels using the standard addition method. The average recovery of quercetin and gallic acid was found close to 99% suggesting the accurateness of the method. The proposed validated high performance thin layer chromatographic method offers a new, sensitive, specific and precise gauge for quantification of quercetin and gallic acid in *L. indica*.

Introduction

Leea indica (Burm. f.) Merr., (Syn.: *L. sambucina* Willd., *L. gigantea* Griff., *L. umbraculifera* Cl., *Stephylea indica* Burm. f.) Merr., Vitaceae, a shrub found to be growing in the forests of tropical and subtropical countries including India, Sri Lanka, Malaysia, Thailand, Andaman and Philippines (Stewart and Brandis, 1874; Gamble, 1956). In traditional systems of medicine the leaf is valued in vertigo and diabetes, while the root as antidysentric, anthelmintic, spasmolytic and sudorific and to treat cardiac and skin diseases (Chatterjee and Prakashi, 1997; Khare, 2007; Rahman et al., 2007). The leaf is reported to possess antioxidant (Saha et al., 2004, Reddy et al., 2012), analgesic (Saifuzzaman et al., 2013), antimicrobial (Srinivasan et al., 2010), phosphodiesterase inhibitory (Temkitthawon et al., 2008), sedative, anxiolytic (Raihan et al., 2011) and cytotoxic (Nurhanan et al., 2008; Emran et al., 2012; Raihan et al., 2012) activities. The plant is reported to contain α-tocopherol, β-amyrin (Saha et al., 2005), lupeol, β-sitosterol, ursolic acid, farnesol, phloridzin, gallic acid (Srinivasan et al., 2008), and quercitrin (Joshi et al., 2013). Flavanoids, kaempferol, quercetin, quercitrin, and mearnsitrin have been reported from other species of genus *Leea* (*L. guineense*) (Beck et al., 2003).

HPTLC is as an acquiescent and commonly used technique for qualitative and quantitative analysis of chemical markers in herbal raw materials. HPTLC has advantages of simplicity, sensitivity, accuracy, and is one the most approached technique for developing fingerprint and marker-based standardization of herbal drugs and is commonly applied not only for the identification, assay and testing for purity but also for stability, dissolution or content uniformity of raw materials and formulated products (Mukherjee et al., 2010).

Taking in to consideration the non-existence of a routine method of analysis, a simple HPTLC method for quantitative estimation of quercetin and gallic acid simultaneously is proposed for *L. indica*. The proposed method was validated by specificity, range, linearity, accuracy, precision, detection limit, quantitative limit, and robustness according to the ICH guidelines (ICH, 1996, 2005).

Materials and methods

Chemicals and solvents

Ethyl acetate, methanol, toluene, HCl, NaOH, distilled water used were of analytical grade. Reference standards quercetin and gallic acid (purity 99.9%) were purchased from Sigma–Aldrich.

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http://dx.doi.org/10.1016/j.bjp.2016.05.017

Plant material

The fresh shrub of *Leea indica* (Burm. f.) Merr., Vitaceae, was procured from Waghai Botanical Garden, Dang, Gujarat in the month of September 2012 and authenticated by the taxonomist of South Gujarat University, Surat, India. Voucher specimen of plant (LM 5412) has been deposited in Department of Pharmacognosy and Phytochemistry, L. M. College of Pharmacy, Ahmedabad. The fresh plant was cut, dried properly, powdered, and passed through 60# sieve. The powdered sample was stored in an airtight container at room temperature.

HPTLC analysis

Quercetin and gallic acid standard stock solutions were prepared by dissolving 2 mg of the compound in methanol in 10 ml volumetric flask and volume was adjusted up to the mark with methanol, from this solution 1–5 μl solutions was applied on precoated TLC plates for quercetin while 1–6 μl was spotted for gallic acid for calibration purpose.

Accurately weighed 10 g of plant powder was extracted exhaustively with ethanol (100 ml × 3) under reflux for 2 h/cycle. Ethanolic extract was evaporated to dryness under vacuum and residue was dissolved in 80 ml aqueous methanol (70%). The aqueous methanol solution was hydrolysed by using 40 ml 2 N HCl and 40 ml toluene and refluxing for 2 h. After neutralizing the solution by 5% NaOH, it was again refluxed for 1 h. The aqueous phase was successively extracted with toluene (2× 30 ml) and ethyl acetate (4× 50 ml). Ethyl acetate extract was evaporated to dryness and 10 mg of extract was dissolved in 5 ml methanol and 15 μl of methanolic solution was applied to a TLC plate. Co-TLC study was conducted using silica gel G 60F254 plate and toluene-ethyl acetate-formic acid, 5:4:1 (v/v/v) as a mobile phase. The plates were observed under UV after derivatizing using Natural products-polyethylene glycol reagent (NP/PEG) for quercetin and 5% $FeCl_3$ for gallic acid.

HPTLC analysis was performed on 10 cm × 10 cm aluminum backed HPTLC plates coated with a 0.2 mm layer of silica gel G $60F_{254}$ (Merck, India). The plates were washed with methanol and activated in an oven at 110 °C for 5 min prior to analysis. The standard and sample solutions were applied to the plates in form of bands of 5 mm long, 1 mm from bottom of the plate and 1 mm from the edge by means of automatic TLC sampler (CAMAG semiautomatic IV). The plates were developed using toluene-ethyl acetate-formic acid 5:4:1 (v/v/v) as mobile phase in CAMAG twin trough chamber, presaturated with mobile phase vapor for 30 min before development. After development, the plate was dried in air for 5 min and scanned immediately at 254 nm using TLC scanner (CAMAG scanner-III). A TLC visualizer was used for the photodocumentation of the plates.

Validation of method

The method was validated as per ICH guidelines. Calibration curve for quercetin and gallic acid were obtained by plotting the peak area against their amount applied to the plate. The limits of detection (LOD) and quantification (LOQ) were determined by equation as per ICH guidelines. Instrumental precision was determined by repeated analysis of standard quercetin and gallic acid. Repeatability was assessed for each reference standard by applying three concentrations of these standards in triplicate on the plate, which were expressed in terms of percentage relative standard deviation (% RSD) and standard error (SE). The intra-day precision was determined at three different concentration levels of different markers, 200, 400 and 600 ng/spot, six times on the same day, and the inter-day precision was determined at three different concentrations of markers, 100, 300 and 500 ng/spot, six times on five different interval days over a period of one week. System suitability was tested by applying 600 ng/spot of quercetin and gallic acid standard solution to the plate seven times and measuring the relative standard deviation (RSD%) for the peak areas and RF. To study the accuracy and precision of the method, recovery studies were performed by the method of standard addition. The recovery of added standard was studied at two different levels, each being analyzed in a manner similar to that described for the assay (Biringanine et al., 2006; Wagner et al., 2008).

Table 1
Validation parameters for quercetin and gallic acid estimation.

Parameters	Results	
	Quercetin	Gallic acid
Correlation coefficient	0.991	0.999
Linearity range (ng)	200–1000	200–1200
Precision (C.V.)		
Repeatability of measurement	0.89	0.359
Repeatability of application	0.53	0.42
Intra day	0.23–0.43	0.2–0.6
Interday	0.97–1.23	0.1–1.13
Limit of detection (ng/spot)	21.31	14.8
Limit of quantification (ng/spot)	64.57	55.04
Accuracy (%)	98.02–99.09	99.28–100.26
Specificity	Specific	Specific

Results and discussion

Quercetin and gallic acid were quantified by HPTLC for the first time in *L. indica*. The identity of quercetin was confirmed by co-chromatography and overlain absorption spectra with reference standard, when scanned at 254 nm. Also the bathochromic shift in UV spectra with $AlCl_3$/HCl relative to methanol spectra asserted presence of quercetin in the plant.

The densitometric chromatogram of HPTLC fingerprint of the ethyl acetate extract is shown in Fig. 1. The peaks resolving at R_f 0.63 and 0.45 in test solution were found to be superimposing with those of respective standards of quercetin and gallic acid. Peak purity was studied by UV overlay spectra obtained from the standards and from the corresponding peaks from test (Fig. 2).

The content of quercetin and gallic acid in plant was calculated on the basis of peak area and was found to be 0.13% and 0.041% (w/w) respectively. The calibration plots indicate that the peak-area response was a polynomial function of the amount of standards quercetin and gallic acid in the range 200–1000 ng and 200–1200 ng respectively. The correlation coefficient was found to be 0.991 and 0.999 with RSD, 0.97–1.23% and 0.1–1.13% for quercetin and gallic acid respectively in the developed method. Intraday and interday precision was calculated for the developed method by performing the analysis at three different levels on same day as well as on different days respectively. RSD for repeatability of measurement of peak area based on 7 times measurement of 600 ng/spot of standards quercetin and gallic acid were found to be 0.89 and 0.359% respectively. RSD for repeatability of measurement of peak area based on a 7 time measurement of 1 μl/spot of sample extract was found to be 0.53 and 0.42% quercetin and gallic acid respectively. The limits of detection (LOD) and limit of quantification (LOQ) were determined to be 21.31 and 64.57 ng/spot for quercetin and 14.86 and 55.04 ng/spot for gallic acid respectively. Percentage recovery of quercetin and gallic acid were found to be in the range of 98.02–99.09 and 99.28–100.26 respectively, which indicated that the developed method was accurate and satisfactory. Summary of all validation parameters for the developed HPTLC method for simultaneous estimation of quercetin and gallic acid are listed in Table 1.

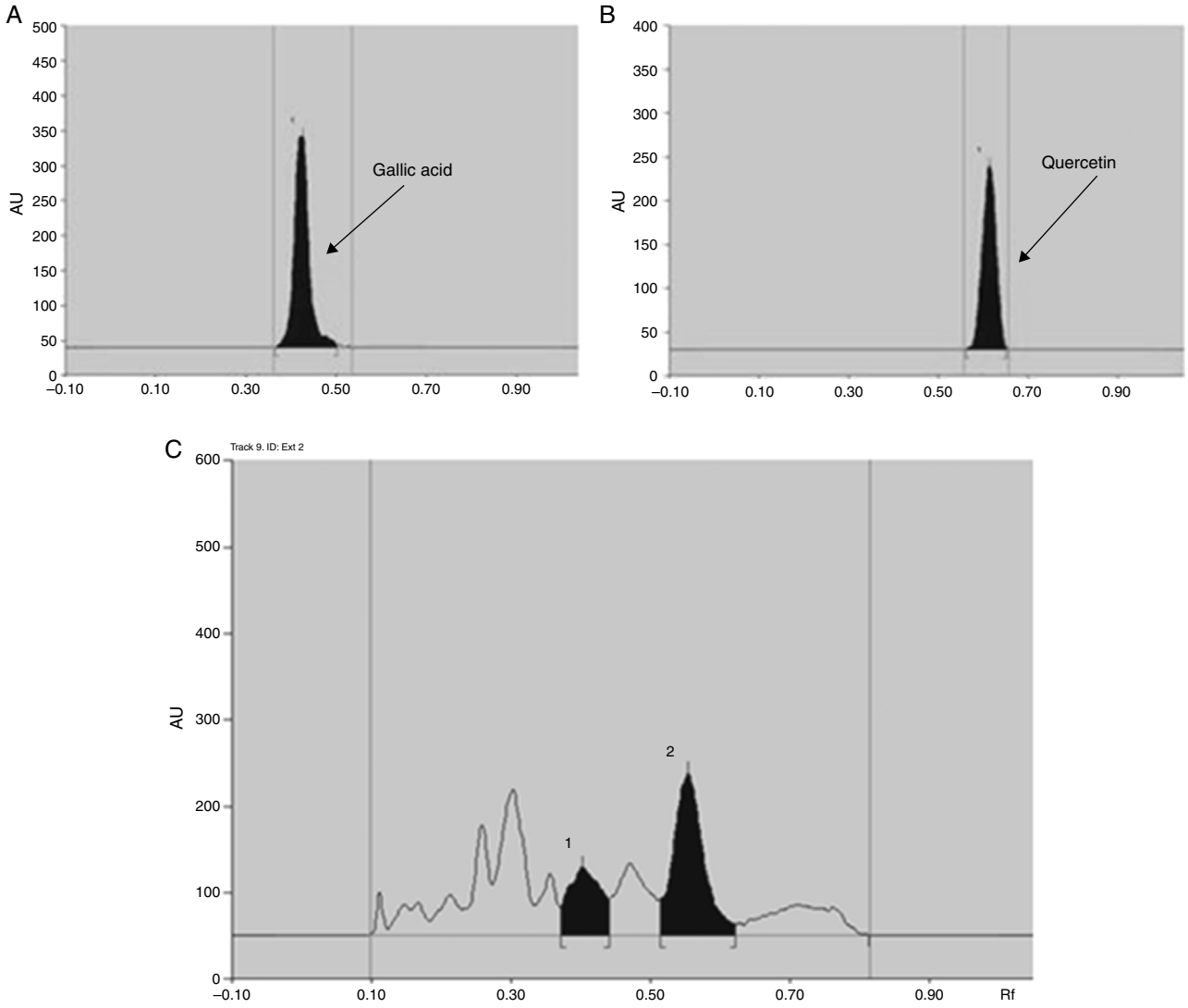

Fig. 1. Densitometric chromatogram of hydrolysed methanol extract of *Leea indica* (A), reference standards gallic acid (B) and quercetin (C) scanned at 254 nm.

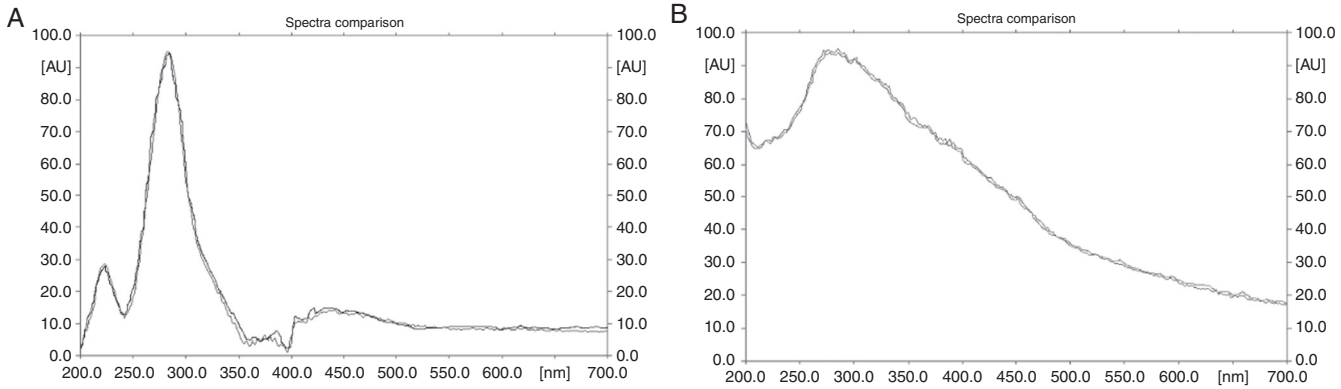

Fig. 2. UV Overlain spectra of gallic acid (A) and quercetin (B) standards and in test solution track scanned at 254 nm.

The method was found to be precise, reliable, and suitable because the recovery in each case was more than 98%. Thus the present HPTLC method enables simple, rapid and accurate quantification of quercetin and gallic acid in plant of *L. indica* simultaneously.

Authors' contributions

AAP (M. Pharm. student) contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and drafted the paper. AAA and

AHP contributed to chromatographic analysis. AAA contributed to critical reading of the manuscript. MBS designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. 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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