International Journal of Phytopharmacology

IJP

HPTLC DENSITOMETRIC QUANTIFICATION OF CAFFEIC ACID AND BOERAVINONE B IN *BOERHAVIA DIFFUSA* LINN

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ABSTRACT

Caffeic acid (3, 4-dihydroxycinnamic acid) is one of the major polyphenolic compounds found in numerous plant species, acts as a primary antioxidant and can be helpful for improving or preventing a number of chronic diseases. Boeravinone B, chemically a retinoid, has been found to inhibit the drug efflux activity of breast cancer resistance protein. Both act as potent antioxidant and anticancer compounds. A new and simple analytical method using HPTLC was developed for the simultaneous estimation and validation of caffeic acid and boeravinone B in one solvent system: toluene, ethyl acetate, formic acid and methanol (3:3:0.8:0.2). The densitometric determination of caffeic acid and boeravinone B was carried out in reflection /absorption mode at 330 nm for caffeic acid and 283nm for boeravinone B respectively. The accuracy of the method was checked by a recovery study conducted at three different levels and the average percentage recovery was found to be 98.8 % for caffeic acid and 98.98 % for boeravinone B. The method was validated according to ICH guidelines. The method reported here is reproducible and can be applied for the quantitative analysis of the above two compounds in the *Boerhavia diffusa* leaves and roots of *Boerhavia diffusa* Linn.

Key words: *Boerhavia diffusa* Linn, HPTLC, caffeic acid, boeravinone B, quantification.

INTRODUCTION

Boerhavia diffusa is an herbaceous member of the Nyctaginaceae family which has a long history of use by indigenous and tribal people of Brazil and India. In particular, roots and leaves of this plant have been widely used in the folk medicine to treat several illnesses including those affecting the gastrointestinal tract. Caffeic acid (CA) and caffeic acid phenethyl ester (CAPE), members of the polyphenolic compounds, are present in high concentrations in medicinal plants. CA and CAPE have been investigated for direct antitumor activity in vivo and in vitro (Joseroberto MP *et al.,* 2012), anti-platelet activity (Chen TG *et al.,* 2007), acute pneumonitis (MiaoFen C *et al.,* 2005), neuroprotective and antioxidant (Chang HJ *et al.,* 2011), antimicrobial and fungi toxicity (Khim PC *et al.,* 2009), anticancer (Hong

YJ *et al.,*1999; Masao H *et al.,* 1997), antioxidant (Wolf W *et al.,* 2004), potent collagen antagonist (George H *et al.,* 2007), antioxidant, antihypertensive, anti-ischemia reperfusion, anti-thrombosis, antihypertension, anbti-fibrosis, antivirus and antitumor (Jiang RW *et al.,* 2005), antihyperglycemic and antioxidant (Jung UJ *et al.,* 2006), cytotoxic effect on human multiple myeloma cell line (Ozgur K *et al.,* 2009), anti-proliferative and apoptotic effect on human breast cancer (Kampa M *et al.,*2004), protective effect on normal and transformed human skin cells (Neradil J *et al.,* 2003; Janbaz KH *et al.,* 2004) antidepressant and anxiolytics (Takeda H *et al.,* 2003), anti-inflammatory and analgesics (Chen YF *et al.,* 1995), myocardial ischemia and apoptic changes (Cagil K *et al.,* 2005) prevents methotrexate-induced hepatorenal oxidative injury (Cakir T *et al.,* 2011) Many retinoids have been isolated from the roots of this plant (Lami N *et al.,*1990; Lami N *et al.,* 1991; Lami N *et al.,* 1991), which showed potent anticancer (Ahmed-Belkacem A *et al.,* 2007), powerful antioxidant and genoprotective properties

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(Aviello G *et al.*), spasmolytic activity (Borrelli F *et al.,* 2006). In ayurveda, it is an important constituent of more than 30 formulations of different categories i.e. Asava, Arista, chyawanprash, churna and tailas according to the formulations described in Bhaisjya ratnavali, Rasratnakara, Bhavprakash nighantu. These formulations have been indicated for several chronic diseases e.g. Jaundice, Splenomegaly, Alimentary tract disorders, Asthma, Renal tract disorders etc. Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost effectiveness and rapidity (Srivastava V *et al.,* 2008; Hajimehdipoor H *et al.,* 2011). In the present paper, we report an HPTLC method that provided simultaneous validation and quantification of caffeic acid and boeravinone B in *Boerhavia diffusa* roots and leaves. Various validation aspects of the analysis, namely peak purity, recovery and limit of detection and quantification, etc., have been measured

MATERIALS AND METHODS
Chemicals and Reagents

All chemicals used were of analytical grade and purchased from Qualigens, Fine chemicals, Mumbai, India. Reference standard boeravinone B and caffeic acid were purchased from Natural Remedies Pvt. Ltd. (Bangalore, India). Methanol, HPLC grade was used as a solvent for the preparation of standard and sample solutions. The entire sample used for the analysis was filtered through 0.22 μm membrane filter.

Chromatographic conditions

An HPTLC plate, 20x10cm(0.2mm thickness) precoated with silica gel 60 F 254 TLC plates (E. Merck) was used. The spotting device was a CAMAG linomat-5 Automatic Sample Spotter (Camag Muttenz, Switzerland); the syringe, 100 μl (Hamilton); the developing chamber was a CAMAG glass twin trough chamber (20×10cm); the densitometer consisted of a CAMAG TLC Scanner-3 linked to win-CATS software; the experimental condition temperature 24 ±2°C, relative humidity 42%.

Preparation of Standard caffeic acid and boeravinone B

Stock solutions were prepared by dissolving 1 mg of standards in 1ml methanol HPLC grade. Stock solution was further diluted to prepare working solutions with methanol in the concentration range 160– 340 ng. Five microliters of each working standard solution was spotted on the pre-coated silica gel 60 F_{254} TLC plates and calibration curve was generated by linear regression based on the peak areas.

Preparation of extracts

Boerhavia diffusa was collected from the campus of Jamia Hamdard, Delhi, India in November 2011, cleaned; shade dried and reduced to coarse powder in a mechanical grinder and passed through sieve no: 12. The powdered roots and leaves were separately subjected to extraction by Soxhlet extraction using methanol for 24 h for isolation of caffeic acid and boeravinone B. Extracts was filtered and concentrated in a rotary evaporator under reduced pressure to get residues. The crude extracts obtained were partitioned with hexane (3×250 ml), chloroform (3×250 ml) and ethyl acetate (3×250 ml). The dried chloroform extract was dissolved in HPLC gade of methanol and finally applied to HPTLC plate.

Preparation of Sample Solutions

Accurately weighed 0.1g of root and leaf extracts were dissolved in 10 ml HPLC grade methanol and sonicated for 10 minutes. The volumes make up with methanol and the solution was filtered through 0.22μ syringe filter before injecting into the HPTLC system.

Calibration Curve for caffeic acid and boeravinone B

Standard solutions of caffeic acid and boeravinone B were applied in triplicate on separate re-coated silica gel 60 F254 TLC plates using a CAMAG Linomat IV Automatic Sample Spotter. The plates were developed in solvent system of toluene: ethyl acetate: formic acid: methanol in a CAMAG glass twin trough chamber (20×10 cm) up to a distance of 8 cm. After development, the plates were dried in air and scanned at 330 and 283 nm using CAMAG TLC Scanner-3 and CATS-4 software. The peak areas were recorded. Calibration curves of caffeic acid and boeravinone B were obtained by plotting peak areas versus applied concentrations of caffeic acid and boeravinone B respectively.

Estimation of caffeic acid and boeravinone B in chloroform extracts

A 5μl each of sample solutions were applied in triplicate on a pre-coated silica gel60 F_{254} TLC plate with CAMAG Linomat IV Automatic Sample Spotter. The plate was developed and scanned as described above. The peak areas and absorption spectra were recorded. The amount of caffeic acid, boeravinone B in *Boerhavia diffusa* roots and leaves were calculated using the respective calibration curves of caffeic acid, boeravinone B.

Method Validation

The method was validated for precision, repeatability and accuracy. Instrumental precision was checked by repeated scanning of the same spots of caffeic acid (100 ng/ band) and boeravinone B (100 ng/ band) seven times each and was expressed as coefficient of

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variance (% CV). The repeatability of the method was affirmed by analyzing 100 ng/band of standard solutions of caffeic acid and 100ng/ band of standard boeravinone B after application of HPTLC plate (n = 7) and analyzing them as described in the generation of calibration plot; the results were expressed as % CV. Variability of the method was studied by analyzing aliquots of standard solutions of caffeic acid (100, 200 and 400 ng/band) and boeravinone B (100ng, 200ng and 400 ng/band) on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy of the method was tested by performing recovery studies three levels (50, 100 and 125% addition). To 0.1g of leaves and root extract of *Boerhavia diffusa*, known amounts of caffeic acid (19 and 22 μg) and boeravinone (17.8 and 17 μg) were added, extracted, and estimated as mentioned in section 2.5 and 2.6. The percent recoveries as well as average percent recovery were calculated. The ratio of mobile phase, temperature, relative humidity was varied in the range of ±5%. The plates were prewashed by methanol and activated at 60±5°C for 5, 10 and 15min respectively, prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied between 0, 30, 60 and 90 min. Robustness of the method was carried out at three different concentration levels: 100, 200, and 400 ng/band. For the determination of the limit of detection and the limit quantification, different dilutions of the standard solution of caffeic acid and boeravinone B were applied along with methanol as blank on the basis of signal to noise ratio.

RESULTS AND DISCUSSION

The method development and selection of a suitable mobile phase involved several trials because of the complexity of the chemical composition of the herbals and the affinities of the components towards various solvents. The solvent system: toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2) gave the best

resolution of caffeic acid (R_f =0.64) and boeravinone B (R_f =0.73) from the other components of the sample extracts, and enabled their simultaneous quantification. The identity of the bands of caffeic acid and boeravinone B in the root and leaves extracts was confirmed by overlapping their absorption spectra with those of the standards (Figure 1 and Figure 2) using CAMAG TLC scanner 3. The purity of caffeic acid and boeravinone B bands in the sample extracts was analyzed by comparing the absorption spectra at start, middle, and end position of the bands. Further, bands of caffeic acid and boeravinone B were detected by spraying with NP-PEG Reagent.The method was validated in term of precision, repeatability and accuracy (Table 1 and 2). The repeatability of sample application and peak area measurement by the HPTLC method, expressed as % CV, was 0.44 and 0.69 for caffeic acid and boeravinone B respectively. The limit of detection and limit of quantification were found to be 40ng, 150ng/ band and 50ng, 150ng/band for caffeic acid and boeravinone B. This indicated that the new method exhibit a good sensitivity for the quantification of caffeic acid and boeravinone B. The quantification of sample extract of caffeic acid and boeravinone b was carried out by plotting a graph between concentration ranges 160-340ng with correlation coefficient of 0.998 and 0.999. Five microliters of each sample solution from different extracts were taken and each one of them was applied on the silica gel 60F$_{254}$ plate in triplicate with a similar pattern. The percentage yield of content of analytes in the sample extracts were calculated on the basis of calibration curves (Table 3). The average percentage recovery at three different levels of caffeic acid and boeravinone B in extracts of *B. diffusa* (leaf and root) were found to be 97.8, 97.23 and 98.92, 98.52 respectively (Table 4). The robustness test for both compounds was carried out by HPTLC and the percentage RSD was found to be less than 2.0%. The average percentage recoveries and low % RSD values demonstrate the reliability and reproducibility of the proposed method.

Fig 1. Densitogram of absorption spectra of Boeravinone (A) and caffeic acid (B) in various samples of root and leaf of *Boerhavia diffusa* with their respective standards
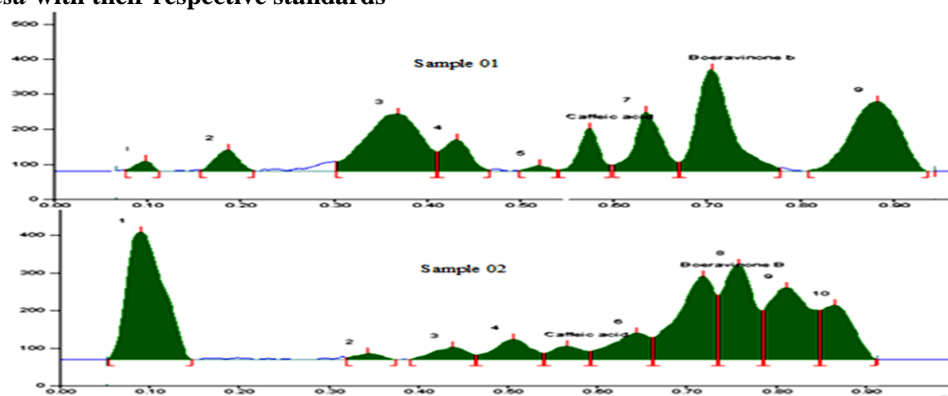
Fig 2. Overlain spectrum of root extracts (sample 01) and leaf extracts (sample 02) of *Boerhavia diffusa* with caffeic acid and boeravinone B standard, scanned at 330nm and 283nm respectively
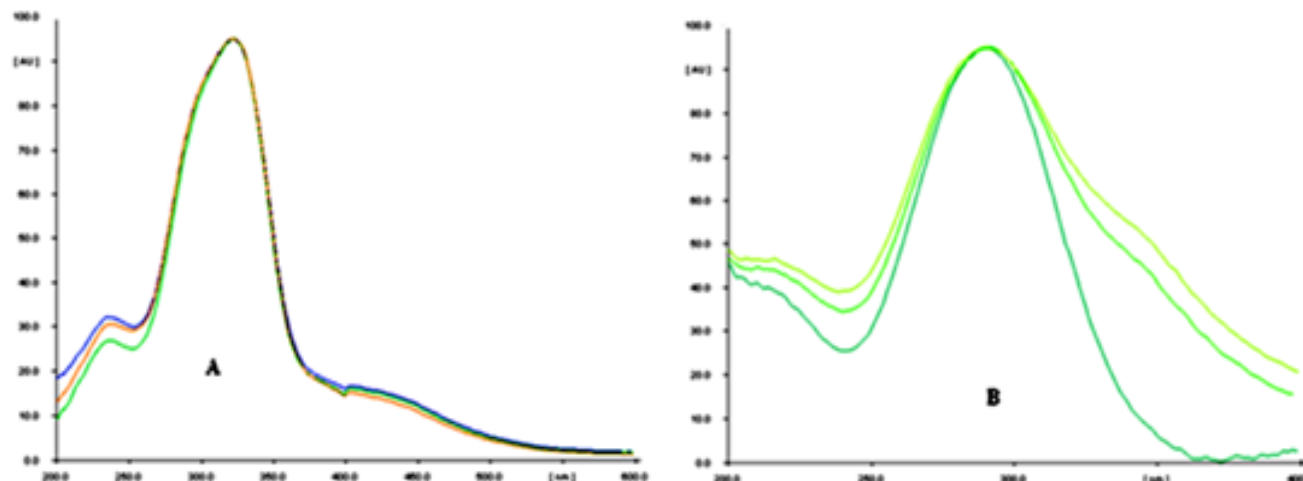

Table 1. Method validation parameters for the estimation of caffeic acid and boeravinone B by HPTLC method

Sr.No	Parameters	Result	
		Cafferic acid	Boeravinone B
1	Instrumental precision (% CV, n = 7)	0.44	0.69
2	Repeatability (% CV, n = 7)	0.41	1.8
4	Limit of detection	40ng	50ng
5	Limit of quantification	150ng	150ng
6	Specificity	Specific	Specific
7	Linearity (correlation coefficient)	0.998	0.999
8	Range (ng /band)	160-340 ng	160-340 ng

Table 2. Intra-day, Inter-day precision of caffeic acid and boeravinone B by HPTLC method

Marker compounds	Concentration (ng/band)	Inter-day precision % RSD(n=3)	Intra-day precision % RSD(n=3)
Caffeic acid	100	0.68	0.91
	200	0.96	1.05
	400	0.88	1.09
Boeravinone B	100	1.3	1.2
	200	1.3	0.84
	400	0.85	0.61

Table 3. Caffeic acid and boeravinone B content found in different sample of *Boerhavia diffusa* by HPTLC method

Tested samples	Content of caffeic acid %w/w Standard deviation (SD, n=3)	Content of boeravinone B %w/w Standard deviation (SD, n=3)
Leaf extract	0.017±0.002	0.022±0.003
Root extract	0.0356±0.0024	0.038±0.007

Table 4. Recovery study of caffeic acid and boeravinone B at 50 % and 100 % addition by the proposed HPTLC method

Markers	Amount of marker present in chloroform extracts (µg)	Amount of marker added (µg)	Amount of marker found (µg)	Recovery (%)	Average Recovery (%)
Root extracts					
Boeravinone B	17.0	8.5	25.31±4.22	99.25	98.92
	17.0	17.0	33.6±3.66	98.82	
	17.0	21.5	38.0±3.21	98.70	
Caffeic acid	220	11.0	32.4±4.77	98.18	98.52
	22.0	22.0	43.3±2.99	98.40	
	22.0	27.5	49.0±4.11	98.98	
Leaf extracts					
Boeravinone B	17.8	8.9	26.0±2.66	97.37	97.8
	17.8	17.8	35.1±4.15	98.59	
	17.8	22.2	39.0±3.99	97.50	
Caffeic acid	19.0	8.0	26.4±3.22	97.77	97.23
	19.0	19.0	37.5.0±4.12	98.68	
	19.0	23.0	40.0±3.23	95.23	

CONCLUSION

This is the first validated report for the simultaneous quantification of two bioactive compounds viz., caffeic acid and boeravinone B in the leaf and root of *Boerhavia diffusa.* The method was found to be simple, precise, specific, sensitive and accurate and can be used for their quantification in the plant materials. It can also be used in routine quality control of herbal raw materials, proprietary and official Ayurvedic formulations.

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