Quantitative Evaluation of *Boerhavia diffusa* and Its Commercial Formulations with Respect to Its Major Bioactive Marker, Eupalitin Galactoside, Using High-Performance Thin-Layer Chromatography

Uppuluri Venkata Mallavadhani*, Yagnambhatla Aparna, Satyabrata Mohapatra, and **Dhanunjay Vithalrao Mane**

Key Words:

Boerhavia diffusa
Eupalitin-3-O-β-D-galactoside
High-performance thin-layer chromatography
Quantification

Summary

In view of extensive applications in the Indian Ayurvedic system of medicine and the increased demand for both as single ingredient and as a major component in multiherbal formulations of *Boerhavia diffusa,* a rapid, sensitive and reproducible high-performance thin-layer chromatography (HPTLC) method has been developed for its quantitative evaluation with respect to its major bio-active marker, eupalitin galactoside. For the separation of the marker compound, HPTLC silica gel F_{254} pre-coated plates were used with *n*-butanol–acetic acid–water (8:1:1) as the mobile phase. The method was validated for limit of detection, limit of quantification, linearity, specificity, precession, and recovery. The results indicate that the bio-active marker was present in 0.075% in *B. diffusa* whole plant. Surprisingly, the marker compound could not be detected even in traces in two of the *B. diffusa* commercial formulations such as punarnava mandur and artin capsules, which contain more than ten herbal and herbo-mineral ingredients. This might be due to the loss of the marker compound during their manufacturing process or different plant parts used in the two test samples. The developed method can be used successfully for the quality control and quality assurance of *B. diffusa* formulations, where the whole plant is used.

1 Introduction

Boerhavia diffusa Linn. (Fam. *Nyctaginaceae*) is a perennial creeping weed found abundantly in India and other parts of the world [1]. It is popularly known as "Punarnava" in the Indian Ayurvedic system of medicine and classified as "Rasayana" herb, which is said to possess properties like helping anti-aging, re-establishing youth, strengthening life and brain power, and preventing diseases which imply that they increase the resistance of the body against immunomodulation [2]. It is also reported in traditional medicine for various ailments such as diuresis, edema, and ascites from early cirrhosis of the liver and chronic peritonitis, jaundice, anasarca, strangury, and gonorrhea. Its root is reportedly used as an analgesic, laxative, and anti-inflammatory agent. Its leaf juice is used in ophthalmia [3]. In Ayurvedic texts, more than 35 formulations of different types containing it as the major ingredient were recorded [4]. It was reported in the literature that the alcoholic extract of the whole plant showed potent hepatoprotective activity against experimentally induced carbon tetrachloride hepatotoxicity in rats and mice. The extract also showed strong choleritic activity [5, 6]. It was also reported that the alcoholic extract of the whole plant exhibited cardiotonic effect in perfused frog heart preparation and anaesthetized dog heart *in situ* [7]. *B. diffusa* plant reported to contain a large number of secondary metabolites such as flavonoids, alkaloids, steroids, and triterpenoids. The major metabolites identified in this plant are punarnavine, borhaavone, boeravinone (A–F), diffusarotenoid, quercetin, kaempferol, eupalitin-3-O-β-D-galactopyranoside, punarnavoside, boerhavine, liriodendrin, boerhavisterol, β-ecdysone, and boeradiffusene [8–10]. In spite of its excellent traditional medicinal uses, significant biological activities attributed to its various extracts and elaborating a large number of secondary metabolites, no rapid, reproducible, and efficacious quantitative method has been reported so far for *B. diffusa* plant. It is noteworthy to mention here that there are a few analytical methods reported for *B. diffusa* based on the markers boeravinones [11] and punarnavoside [12, 13]. However, these methods are valid only for the root part but not for the whole plant material, which is actually the official drug traditionally known as "punarnava". Critical analysis of the compounds isolated from *B. diffusa* and their biological activities reveals that eupalitin-3-O-β-D-galactopyranoside was found to exhibit similar immunomodulatory activity [14] as that of the plant. Furthermore, eupalitin-3-O-β-D-galactopyranoside was found to accumulate in significant levels in the whole plant of *B. diffusa*. Hence, it can be considered as the bio-active marker compound for the

U.V. Mallavadhani, Y. Aparna, and S. Mohapatra, Natural Products Chemistry Division, Council of Scientific and Industrial Research–Indian Institute of Chemical Technology (CSIR–IICT), Hyderabad 500007, India; S. Mohapatra, present address: R & D Centre, Emami Limited, Kolkata 700056, India; and D.V. Mane, UG.PG and Research Center, Department of Chemistry, Shri Chhatrapati Shivaji College, Osmanabad 413606, India.
E-mail: mallavadhani@iict.res.in

quantitative evaluation of *B. diffusa* and some of its commercial formulations. In connection with our several research programs on botanical and Ayurvedic drugs, we found that high-performance thin-layer chromatography (HPTLC) is a highly useful analytical tool for their quantitative evaluation [15, 16]. With this background, we have now developed an efficient HPTLC method for the quantitative evaluation of *B. diffusa* whole plant based on its major bio-active marker compound, eupalitin-3-O-β-D-galactopyranoside, and the results are presented herein.

2 Experimental

2.1 Plant Material

The whole plant material of *B. diffusa* was procured through IIIM (formerly RRL, Jammu, India) and its identity was further confirmed by taxonomists.

2.2 Chemicals, Solvents, and Commercial Formulations

For extraction and isolation of the marker compound, commercial grade solvents such as *n*-hexane, ethyl acetate, acetone, and methanol were used after drying and distillation following standard purification procedures. Silica gel G and silica gel (100–200 mesh) of ACME (Mumbai, India) grade were used for thin-layer chromatography (TLC) and column chromatography, respectively. Precoated HPTLC silica gel 60 F_{254} (E. Merck, Darmstadt, Germany) alumina backed plates were used. Two commercial formulations of *B. diffusa* such as punarnava mandur and artrin capsules of M/s Baidyanath (Kolkata, India) and Imis Pharmaceutical (Vijayawada, India), respectively, were procured from the local market.

2.3 Extraction and Isolation of Marker, Eupalitin-3-O-β-D-Galactopyranoside

The powdered plant material (4 kg) was extracted with 50% aqueous ethanol in an aspirator at room temperature for 16 h. The process was repeated three times. The combined solubles on concentration under reduced pressure yielded a reddish brown extract (450 g, 11.25%). The extract showed positive tests for phenols, steroids, and glycosides. The extract showed well-resolved spots in two different solvent system, *viz., n*-butanol–acetic acid–water (8:1:1) and ethyl acetate–methanol–water (6:2:2), in thin-layer chromatography. In order to isolate the marker compound, the extract (80 g) was subjected to detailed column chromatographic separation over silica gel using chloroform–methanol mixtures as eluent. Based on the TLC nature of the column fractions, they were regrouped into four major fractions A–D. These fractions on repetitive column chromatographic purifications followed by recrystallization furnished a pale yellow amorphous powder (1.8 g, 0.25%), m.p. 179°C, R_F: 0.6 (*n*-butanol–acetic acid–water 8:1:1). It gave positive tests for phenols and glycosides. IR (KBr, cm^{-1}): 3446 (hydroxyl), 1658 (carbonyl); ^{1}H-NMR (400 MHz, DMSO-d_6): δ 12.52 (1H, br. s, 5-OH), 10.15 (1H, br. s, 4′-OH), 8.06 (2H, d, J = 8.9 Hz, H-2′, H-6′), 6.84 (1H, s, H-8), 6.81 (2H, d, J = 8.9 Hz, H-3′, H-5′), 5.38 (1H, d, J = 7.7 Hz), 5.12-4.39 (sugar protons), 3.86 (3H, s, 7-OMe), 3.68 (3H, s, 6-OMe). ^{13}C-NMR (100 MHz, DMSO): 177.7 (C-4), 159.9 (C-7), 158.6 (C-2), 156.6 (C-8a), 151.5 (C-5), 133.1 (C-3), 131.6 (C-6), 130.9 (C-2′, C-6′), 120.7 (C-1′), 115.0 (C-3′, C-5′), 105.2 (C-1″), 101.4 (C-6a), 91.2 (C-8), 75.7 (C-5″, C-3″), 73.0 (C-2″), 71.1 (C-4′), 67.8 (C-6′), 56.4 (C-7OCH_3), 60.9 (C-6 OCH_3), ESIMS (positive): 515.30 $[M+Na]^+$ corresponding to $C_{23}H_{24}O_{12}Na$.

2.4 Extraction and Sample Preparation

The powdered material (1.00 g each) was extracted in Soxhlet extractor for 7 h in ethanol. These extracts were concentrated and further dried in a lyophiliser. An amount of 100 mg of each extract was dissolved in 10-mL volumetric flasks.

2.5 HPTLC Instrumentation and Chromatographic Conditions

2.5.1 HPTLC Instrumentation

Chromatographic estimation was performed by spotting standard and extracted samples of *B. diffusa* on pre-coated silica gel aluminum sheet plate 60 F_{254} (20 cm × 10 cm with 250 mm thickness; E. Merck, Darmstadt, Germany) using a CAMAG Linomat V sample applicator (CAMAG, Muttenz, Switzerland) and a 100-mL syringe. The samples, in the form of bands of length 6 mm, were spotted (15 mL) 10 mm from the bottom, 35 mm from left margin of the plate, and 13 mm apart, at a constant application rate of 80 nL s^{-1} using nitrogen aspirator. The plates were developed using the mobile phase consisting of *n*-butanol–acetic acid–water (8:1:1, *v/v*). Linear ascending development was carried out in a 20 cm × 10 cm twin-trough glass chamber (CAMAG) equilibrated with the mobile phase. The optimized chamber saturation time for the mobile phase was 10 min at room temperature. The length of chromatogram run was 5 cm. Approximately an amount of 20 mL of the mobile phase (10 mL in the trough containing the plate and 10 mL in the other trough) was used for each development which required 15 min. It results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, the TLC plates were dried in a current of air with the help of an air-dryer for 5 min. The slit dimension settings of length 5.00 mm and width 0.45 mm and a scanning rate of 20 of mm s^{-1} were employed. The monochromator band width was set at 20 nm.

Densitometric scanning was performed on a CAMAG TLC Scanner III in the absorbance mode at 274 nm and operated by winCATS planar chromatography version 1.1.3.0 (CAMAG).

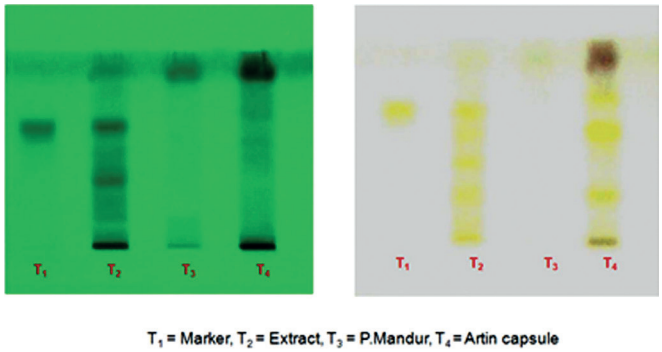

Figure 1

TLC chromatograms of *B. diffusa* and its commercial formulations.

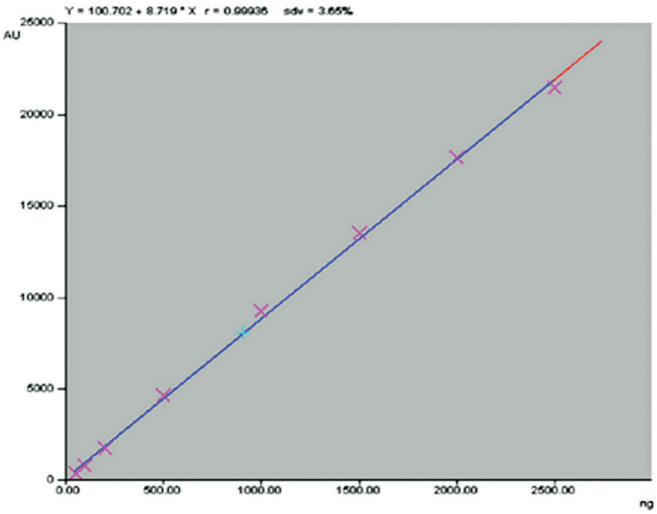

Figure 2

Calibration curves of the marker and its commercial samples.

The sources of radiation utilized were deuterium and tungsten lamp. The concentrations of the compound chromatographed were determined from the intensity of absorbance. Evaluation was *via* peak areas with linear regression.

2.5.2 Visualization

Visualization was done at 254 nm and also after derivatization with freshly prepared vanillin–H_2SO_4 reagent **(Figure 1).**

2.5.3 Calibration Curves of Standard

The calibration curves of the standard in ethanol-containing concentrations of 0.005 mg to 0.25 mg mL^{-1} were prepared from the stock solution of 1.0 mg mL^{-1}. An amount of 10 µL from each solution was spotted on the TLC plate to obtain a final concentration range of 0.05–2.5 µg $spot^{-1}$. The data of peak area *versus* sample concentration were treated by linear regression analysis **(Figure 2).**

2.6 Method Validation

The HPTLC method developed was validated for the following parameters.

2.6.1 Limits of Detection and Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated for the marker compound on the basis of three and ten times the noise level, respectively. LOD was determined

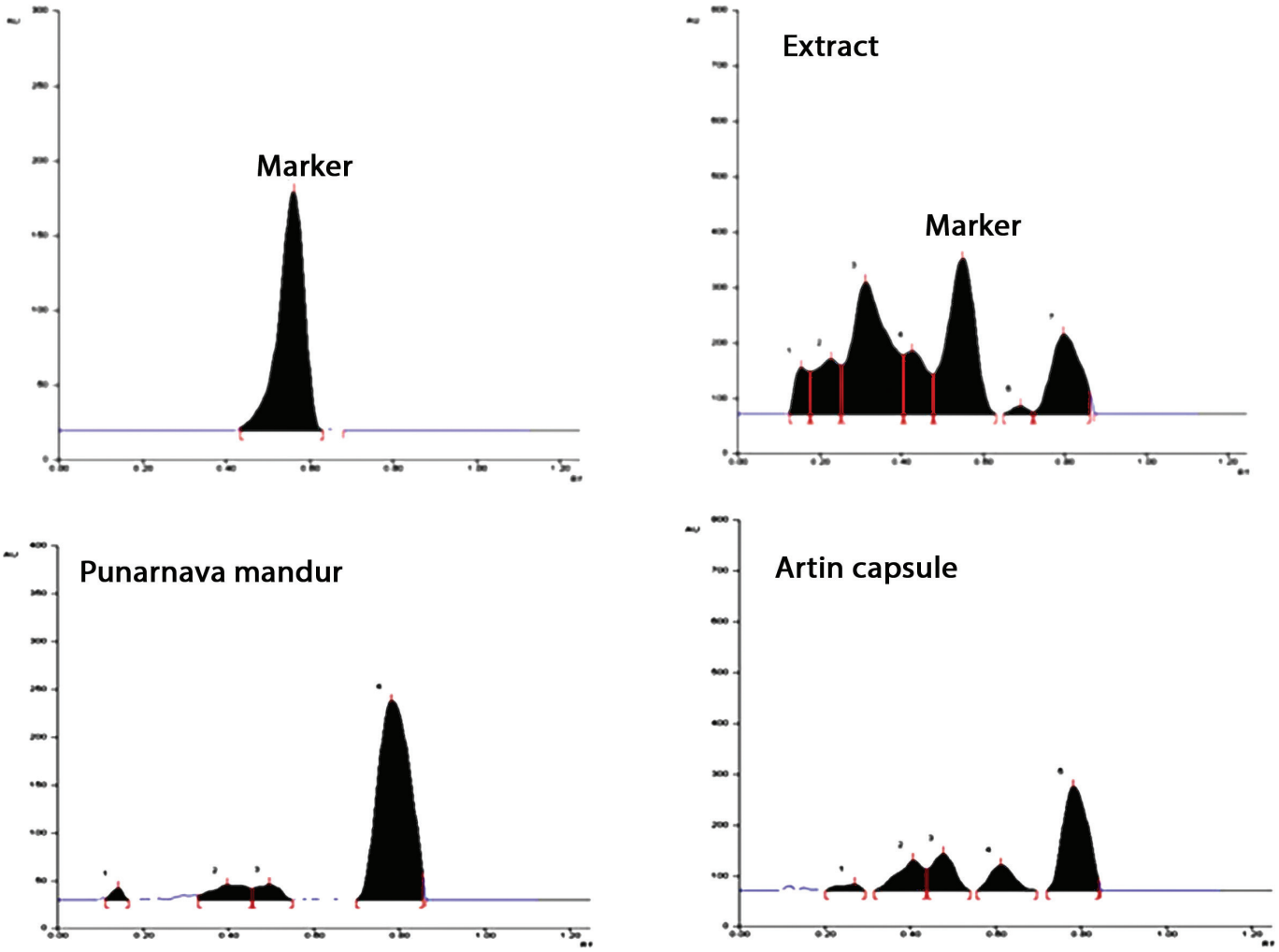

Figure 3

HPTLC chromatograms of the marker compound, *B. diffusa* and its commercial formulations.

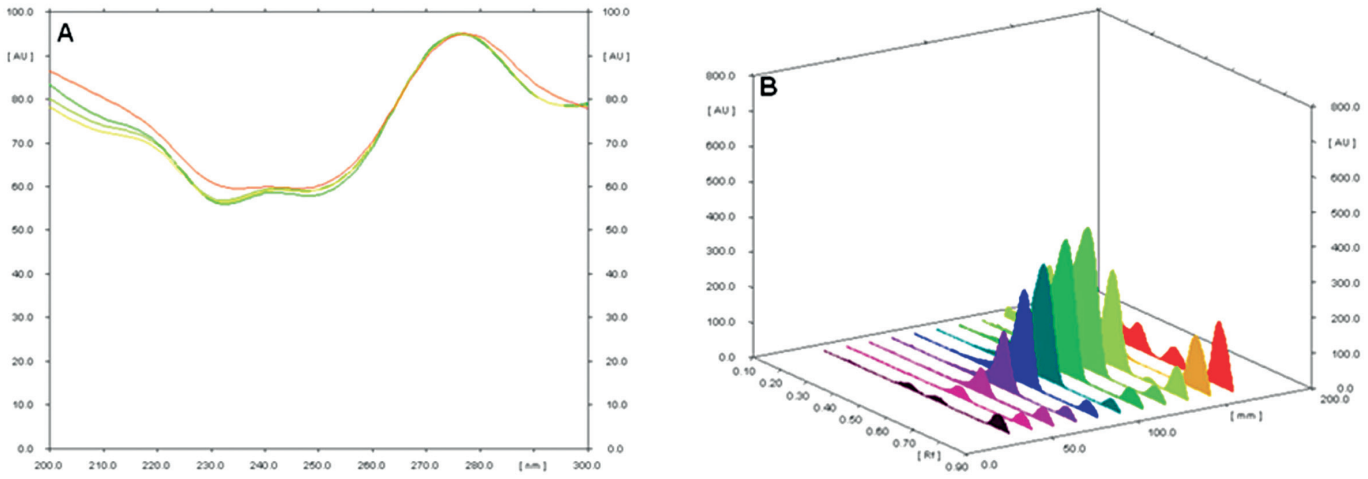

Figure 4

UV spectra (A) and 3D overlaid spectra (B) of the standard and *B. diffusa* samples.

as 3 times the average noise value of blank ethanol applied (10 µL, 5 times). Thus, LOD was calculated as 1.38 ng and LOQ was calculated as 4.18 ng.

2.6.2 Specificity

The specificity of the method was ascertained by analyzing standard and test samples. The band for the marker compound in the test samples was confirmed by comparing the R_F and the spectra of the spot with those of the standard **(Figure 3).** The peak purity of the marker compound was assessed by comparing the spectra at peak start, peak apex, and peak end of the band, respectively. Representative overlaid spectra of the standard and *B. diffusa* extracts are shown in **Figure 4.**

2.6.3 Precision

The repeatability of the measurement ($n = 6$) of the peak area for the compounds was expressed in terms of percent coefficient of variation (*%RSD*). The intra- and inter-day variation study in the analysis was carried out at three different concentration levels.

2.6.4 Recovery

Accuracy was assessed with the method of standard additions at three concentration levels. Accuracy was assessed by comparing the results with the analysis of the standard reference material over the range of 80%–120% of the amount corresponding to the midpoint of the curve.

3 Results and Discussions

The whole plant material of *B. diffusa* was exhaustively extracted with 50% aqueous ethanol, and the resultant extract on extensive Si gel chromatographic purifications resulted in the isolation of the bio-active marker compound as pale yellow amorphous powder. Preliminary physical and chemical tests identified the compound as flavanol glycoside. Detailed spectroscopic analysis (infrared [IR], proton nuclear magnetic resonance [^{1}H-NMR], carbon-13 nuclear magnetic resonance [^{13}C-NMR], and mass spectrometry) confirmed its structure as eupalitin-3-O-β-D-galactopyranoside. Detailed thin-layer chromatographic studies on this compound optimized the solvent system as *n*-butanol–acetic acid–water (8:1:1) which showed a single spot at R_F of 0.55 (Figure 1). This compound was taken as the marker compound and it was quantitatively evaluated from *B. diffusa* whole plant along with two of its commercial samples such as punarnava mandur (Baidyanath) and artrin capsules (Imis Pharma) using HPTLC.

3.1 Method Validation

3.1.1 Sensitivity

The sensitivity of the method was determined with respect to LOD, LOQ, linearity range, and correlation coefficient. Solutions containing 0.05–2.5 µg of standard were spotted on TLC plates. LOD was calculated as 1.38 ng (3 times the noise level), and LOQ was calculated as 4.18 ng (10 times the noise level). The regression data for the four samples are given in the Table 1, which shows good linear relationship in the range studied.

Table 1

Results of linearity.

Linearity range	r^2	Slope	Intercept
0.05–2.5 µg	0.99872	8.719	100.702

3.1.2 Recovery Study

The recovery of the standard was calculated by spiking 1.0 mg to the formulation, punarnava mandur (1.00 g), and extracted in ethanol and analyzed three times as described in Section 2.4. The recovery was calculated by comparing the resultant peak areas with those of the standard (Table 2).

Table 2

Recovery and *RSD* of method.

Sample	Amount spotted [ng]	Amount detected [ng] (mean ± SD, $n = 3$)	*RSD* [%]	Recovery [%]
PM	500	491.54 ± 5.32	1.08	98.30

3.1.3 Precision and Accuracy

Different amounts of punarnava mandur (spiked) samples were spotted on TLC plate. These spots were analyzed by using the above described HPTLC method (Table 3). Precision was expressed as the percent relative standard deviation (*%RSD*).

Table 3

Results of precision and accuracy.

Actual amount of standard spotted [ng]	Amount detected [ng ± SD; n = 5]	*RSD* [%]
300	294.57 ± 4.75	1.61
500	587.24 ± 9.39	1.59
1000	972.46 ± 19.57	2.01

3.1.4 Reproducibility

The repeatability was evaluated by analyzing the known amounts of punarnava mandur (spiked) samples spotted on TLC plate in replicates (n = 5). The inter-day and intra-day precisions were evaluated by analyzing the same amount of analyte over a period of 3 days (n = 5) and expressed in terms of *%RSD*.

Table 4

Results of reproducibility.

Amount spotted [ng]	Amount detected (ng ± SD)	*%RSD*
Inter-day (n = 3)		
300	281.39 ± 5.17	1.83
500	573.36 ± 11.48	2.00
1000	972.64 ± 22.71	2.33
Intra-day (n = 5)		
300	287.65 ± 4.92	1.71
500	585.36 ± 9.47	1.61
1000	977.18 ± 18.84	1.93

3.2 Sample Analysis

The validated HPTLC method was applied for quantitative determination of the marker, eupalitin-3-O-β-D-galactopyranoside, in *B. diffusa* whole plant sample and two of its commercial formulations such as punarnava mandur and artrin capsules, and the results are presented in Table 5. The marker compound was found to accumulate in 0.075% in the *B. diffusa* whole plant sample. Surprisingly, it was found absent in both commercial samples. In order to rationalize these observations, the composition of the two commercial samples were closely observed. Both the commercial samples were found to contain about ten herbal or herbomineral ingredients. Furthermore, it was observed that in both of the commercial formulations, it is the root part of *B. diffusa* that was used. Hence, the marker compound in these two formulations might be lost during their manufacturing process or due to different plant parts used. However, the marker compound could be detected accurately in the whole plant samples. The developed method can be used successfully for the quality control and quality assurance of *B. diffusa* formulations where the whole plant is used.

Table 5

Accumulation of the marker in various *B. diffusa* samples.

S. No.	Samples	Eupalitin galactoside [%]
1	Extract	0.075
2	Punarnava mandur	Not detected
3	Artin capsule	Not detected

4 Conclusion

In the present study, eupalitin-3-O-β-D-galactopyranoside, the bioactive marker of *B. diffusa* was isolated and quantitatively estimated in the whole plant material and two of its commercial formulations by employing the validated HPTLC method. The method is rapid and reproducible. The marker compound was found to be present in 0.075% of the whole plant of *B. diffusa*. The developed method can be successfully employed for the quality control and quality assurance of *B. diffusa* formulations where the whole plant part is used.

Acknowledgment

The authors wish to thank the director of CSIR–IICT, Hyderabad, India, for support and constant encouragement.

References

[1] *A.N. Sahu, L. Damiki, G. Nilanjan, S. Dubey,* Pharmacogn. Rev. **2** (2008) 14–22.

[2] *R. Govindarajan, M. Vijayakumar, P. Pushpangadan,* J. Ethanopharmacol. **99** (2005) 165–178.

[3] *S. Dev* (ed.), A selection of Prime Ayurvedic Plant Drugs, Ancient–Modern Concordance, Anamaya Publishers, New Delhi, 2006.

[4] *S. Mishra, V. Aeri, P.K. Gaur, S.M. Jachak,* BioMed Res. Int. **2014** (2014) 1–19.

[5] *A.K.S. Rawat, S. Mehrotra, S.C. Tripathi, U. Shome,* J. Ethnopharmacol. **56** (1997) 61–66.

[6] *B.k. Chandan, A.K. Sharma, K.K. Anand,* J. Ethanopharmacol. **31** (1991) 299–307.

[7] *R.P. Singh, K.P. Shokala, B.L. Pandey, R.G. Singh, S.R. Usha,* J. Res. Ind. Med. **11 (**1992) 29–36.

[8] *F. Ferreres, C. Sousa, M. Justin P. Valentao, P.B. Andrade, R. Llorach, A. Rodrigues, R.M. Seabra, A. Leitao,* Phytochem. Anal. **16** (2005) 451–458.

[9] *F. Borrelli, N. Milic, V. Ascione, R.Capasso, A.A. Izzo, F. Capasso, F. Petrucci, R. Valente, E. Fattorusso, O. Taglialatela-Scafati,* Planta Med. **71** (2005) 928–932.

[10] *J. Gupta, M. Ali,* Indian J. Chem. Sec. B **37** (1998) 912–917.

[11] *K. Bairwa, A. Srivastava, S.M. Jachak,* Phytochem. Anal. **25** (2014) 415–420.

[12] *R.K. Seth, M. Khanna, M. Chaudhary, S. Singh, J.P.S. Sarin,* Indian Drugs **23** (1986) 583–584.

[13] *J. Lalla, P. Hamrapurkar, D.H. Mamania,* J. Planar Chromatogr. **16** (2003) 465–468.

[14] *R. Pandey, R. Maurya, G. Singh, B. Sathiamoorthy, S. Naik,* Int. Immunopharmacol. **5** (2005) 541–553.

[15] *U.V. Mallavadhani, S. Mohapatra,* J. Planar Chromatogr. **27** (2014) 255–259.

[16] *K. Haribabu, M. Ajitha, U.V. Mallavadhani,* J. Anal. Bioanal. Tech. **5** (2014) 1–6.

Ms received: June 2, 2017
Accepted: September 26, 2017