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Research Article

Phytochemical investigation for the authentication of Indian traditional herbal drug *Boerhavia diffusa* and its differentiation from morphologically similar plant *B. erecta*

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Abstract

Boerhavia diffusa is an important herbal ingredient of several Ayurvedic and Unani formulations and it is commonly called as *Punarnava* in Indian system of medicine. But, this plant is often confused with *Boerhavia erecta* due to morphological similarity and also being adulterated in Indian herbal market. In order to differentiate these plant drugs, in the present work we have developed chemical fingerprints through phytochemical analysis, UV-Visible, FT-IR & NMR spectroscopy, HPTLC and HPLC chromatography. Phytochemical analysis of methanolic extract of investigated materials showed that *B. diffusa* contained higher level of total phenolic compounds (339.40 mg GAE / 100 g) when compared to *B. erecta* (269.87 mg GAE / 100 g). Scanning in UV-Visible spectroscopy exhibited maximum absorption at 212, 362, 428 & 663 nm for *B. diffusa* while *B. erecta* showed peaks at 214, 308, 349, 532 & 663 nm. Similarly, FT-IR spectroscopy of raw materials indicated notable difference between the samples at 400 – 1000 cm^{-1} region. NMR spectroscopy illustrated remarkable difference between the chloroform extract of selected samples at 1-3 ppm region. HPTLC profile of methanolic extract of *B. diffusa* exhibited a total number of 9 peaks while *B. erecta* showed 12 number of peaks. Chemical profile investigated in the present study will be helpful to differentiate *B. diffusa* from *B. erecta* and also useful to prevent their adulteration in herbal industry.

Keywords: *Boerhavia diffusa; B. erecta*; Herbal adulteration; Chemical fingerprints; Quality control.

1. Introduction

India ranks fourth in Asia and tenth in the world in plant biodiversity [1]. About 15,000-20,000 plants have good medicinal value, out of which in India traditional communities use 7000- 7,500 for their medicinal values [2]. The Indian subcontinent has a rich repository of medicinal plants that are used by various indigenous health care systems [3]. Because of fact that herbal medicines are safer than synthetic drugs, demand of medicinal plants has increased many folds in the national and international markets to meet the increasing needs of traditional medicines [4]. As cultivation of medicinal plants in India is limited, over 90% medicinal plants are being harvested from the wild habitats to meet ever-growing demands of national and international market. But, due to various factors natural habitats of medicinal plants are destroying. As a result, natural population of medicinal plants is depleting. Consequently, availability of these medicinal plants is decreasing. This has resulted in a gap in demand and supply, leading to adulteration and substitution for genuine material. One of the examples for such case is adulteration of *Boerhavia diffusa* a well known Indian

traditional medicinal plant with its related species *B. erecta* due to morphological resemblance.

Boerhavia diffusa is an indigenous plant and it is described as Punarnava by an Indian system of medicine [5, 6]. Though considered as a weed, its leaves, seeds and roots are edible and has considerable levels of carbohydrates and proteins [7]. Roots and whole plants of *B. diffusa* are used in the Ayurvedic and Unani systems of medicine [8]. It acts as diuretic, emetic, expectorant, laxative, stomachic, fibrinolytic, anti-convulsant and carminative [9 - 13]. It is used for the treatment of asthma, oedema, anaemia, jaundice, diabetes and liver disorders in indigenous medicine in India [14, 15]. Scientifically it has proven to possess anti-inflammatory [16], anthelmintic activity [17], immune-modulatory effect [18], cardio-protective [19] and anti-cancer [20] properties. Phytochemical profile including pharmaceutically important alkaloids (lunamarine, Punarnavine 1 and 2) and rotenoids (Boeravinones G and H) has been reported in *B. diffusa* [21 – 24].

Boerhavia erecta is a weedy herb of the family Nyctaginaceae and is commonly available in India. Stems of *B. erecta* typically grow to about 60 centimetres tall and 3 mm across, cylindrical, green in colour tinted with purple, and the base is glabrous and woody. Leaves are fleshy, opposite, unequal, 1.5–2.5 cm long, with a petiole. The leaf blade is ovate, upper surface is green and pubescent, and the underside is grayish-white. The inflorescence is cymose, two leafy bracts subtend each branch of the inflorescence, but detach at an early stage. Each peduncle bears 2–6 sessile flowers at its apex. The flowers are tiny, pink and cream. Anthocarps (false fruits) are circular, flat and glabrous. It has been found to possess diuretic action, anti-inflammatory, anti-fibrinolytic, anticonvulsant, anti-microbial, hepatoprotective activities [25] and antioxidant activities [26]. Phytochemical investigation revealed the presence of phenolic and flavonoids in *B. erecta* [27].

Few molecular approaches like PCR-RFLP technique [28] and DNA barcoding [29] have been investigated to differentiate *B. diffusa* from its related species. However, chemical markers are useful in routine analysis and also authentication of the plant material in batch-wise production. Hence, in the present study, we have made an attempt to develop chemical fingerprints to differentiate *B. diffusa* from its adulterant *B. erecta* by using modern analytical tools.

2. Materials and methods

2.1 Sample preparation

Whole plant materials of *B. diffusa* and *B. erecta* were collected from SASTRA herbal garden, Thachenkuruchi, Thanjavur. Both the plant materials were identified and authenticated by the Botanist (Dr. N. Ravichandran) from Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA University, Thanjavur. Plant materials were shade dried and milled into fine powder and used for further analysis.

2.2 Preparation of extracts

Both chloroform and methanolic extracts were prepared by taking 25 g powdered material of each sample separately with 250 ml of respective solvent in a closed glass container and kept on an orbital shaker at 500 rpm for 3 h at room temperature. The contents were then filtered through Whatman filter paper and the final volume was noted. Both chloroform and methanolic extracts were evaporated to dryness using rotovapor (Make: Buchi, Model R-300) and the dry extract was re-dissolved in respective solvents in the ratio of 10 mg/ml. Chloroform extract was used to carry out proton NMR analysis whereas methanolic extract was used for the analysis of phytochemical (polyphenols), UV-Visible scanning, FT-IT and HPTLC finger-printing.

2.3 Quantification of polyphenols

Total phenolic content of methanolic extract of *B. diffusa* and *B. erecta* were analyzed using Folin-Ciocalteu reagent method with some modifications [30]. The extract (100 µl) was added to 250 µl of Folin-Ciocalteau reagent and vortexed for 1 min. Then, 1.0 ml of 5% sodium carbonate solution was added and the mixture is vortexed again for 1 min. A blank was prepared with 100 µl of the solvent (distilled water) instead of the extract. The tubes were incubated at 40°C for 30 min in the dark. The absorbance was read at 720 nm against the blank using Spectrophotometer (Make: Perkin-Elmer). A calibration curve was prepared with standard gallic acid (16 – 100 mg/L, R^2 = 0.9939) and used to calculate the total phenolic content of extracts and the results are expressed as gallic acid equivalents (mg GAE / 100 g).

2.4 Spectroscopic analysis

UV-Visible scanning of suitably diluted methanolic extract of *B. diffusa* and *B. erecta* was carried out in the wave length range of 200 – 780 nm in a UV-Visible spectroscopy (Make: Thermo Scientific Model: Evolution 201).

For FT-IR spectroscopic analysis, finely powdered raw materials of *B. diffusa* and *B. erecta* were oven dried at 60^{o}C. Two milligrams of the sample was mixed with 100 mg KBr (FT-IR grade) and then compressed to prepare a salt disc (3 mm diameter). The disc was immediately kept in the sample holder and FT-IR spectra were recorded in the absorption range between 400 and 400 cm^{-1} using FT-IR spectrometer (Make: Perkin-Elmer, Model: Spectrum-100).

The 1H NMR spectra of chloroform extract of *B. diffusa* and *B. erecta* (5 mg each) in chloroform-d (Sigma-Aldrich, USA) were acquired using a NMR spectrometer

(Make: Bruker Biospin, Switzerland, Model 300 MHz AVANCEII) equipped with a 5 mm BBO probe. The experiments were recorded at 298.15 K using the standard pulse sequence library of Top Spin 1.3 followed by processing of the data by using Top Spin 3.2 software.

2.5 Chromatographic analysis

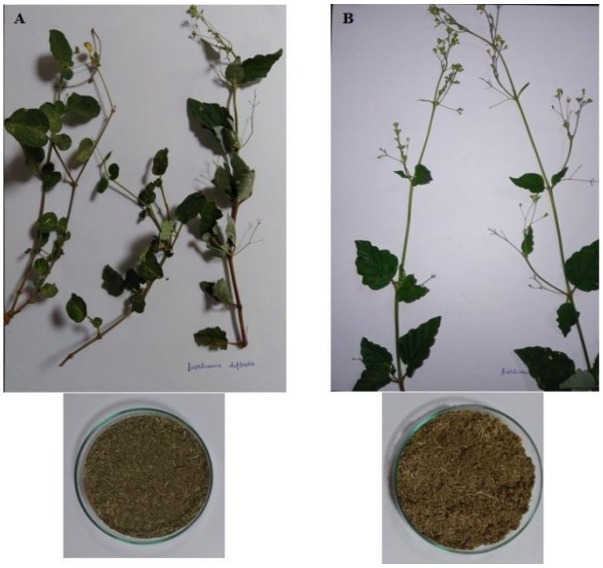
HPTLC profile of methanolic extract of *B. diffusa* and *B. erecta* was analyzed on a pre-coated silica gel 60 F-254 (0.2 mm thickness) HPTLC plate (10 x 10 cm, Merck, Germany). Samples (10 µl each) were applied on the plate as 7 mm bands, 15 mm apart from the edges of the plate, with a Camag Linomat V sample applicator. Mobile phase of Toluene: Ethyl acetate: Formic acid (50:40:10, v/v/v) was used for the analysis. The plates were developed to a distance of 80 mm at 25 ± 5°C in a Camag twin trough glass chamber. The saturation time was 30 min and after development, plates were dried in a hot-air oven, viewed in a Camag UV chamber at 254 and 366 nm and the chromatograms were scanned with a Camag TLC Scanner. The Rf values and fingerprint data were recorded using WINCATS software.

For HPLC analysis, methanolic extract of *B. diffusa* and *B. erecta* were purified using membrane filter (Nupore, PVDF syringe filter 0.45 micron) and analyzed using HPLC (Make: Agilent, Model: Infinity 1200). Mobile phase consists of Acetonitril (A) and Water with 2% acetic acid (B) and the gradient conditions were: 70% B during 0 – 10 min, 60% B in 10 – 15 min, 50% B in 15 – 20 min and 70% B in 20 – 25 min with the flow rate of 1 ml / min. Sample volume of 25 ml was injected manually into the C-18 analytical column (Zorbax Eclipse plus, 250 x 4.6 mm and 5 micron) to separate the phyto-constituents and the output was detected at 320 nm during the run time of 25 min.

3. Results and discussion

Both *B. diffusa* and *B. erecta* has many morphological similarities, but can be distinguished using botanical characters. However, in both the plants the above-ground parts are harvested and sold in market as powdered materials, so it is very difficult to distinguish these plants in powdered drug form (Fig. 1). Hence, based on the availability, location and season, the commercial vendors could collect either *B. diffusa* or *B. erecta* and sell in the market in dried powder form. In this context, development of chemical fingerprints is necessary for quality control of *B. diffusa* drug in Indian herbal industry and also to authenticate the proper drug.

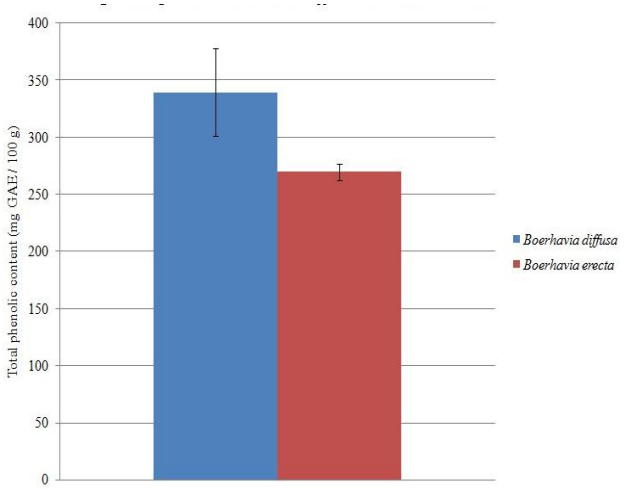
Figure 1: Plant morphology of *Boerhavia diffusa* (A) and *Boerhavia erecta* (B)



3.1 Polyphenolic content

Analysis of total phenolic content of methanolic extract of selected plant materials revealed that *B. diffusa* contained higher level of total phenolic compounds (339.40 mg GAE / 100 g) when compared to *B. erecta* (269.87 mg GAE / 100 g) (Fig. 2). Higher content of polyphenols observed in methanolic extract of *B. diffusa* of the present analysis provides scientific evidence for the better efficacy noted in traditional medicine. Quantification of total phenolic compounds using Folin's-Ciocaltue reagent (Spectrometric method) could help us to differentiate *B. diffusa* from *B. erecta*.

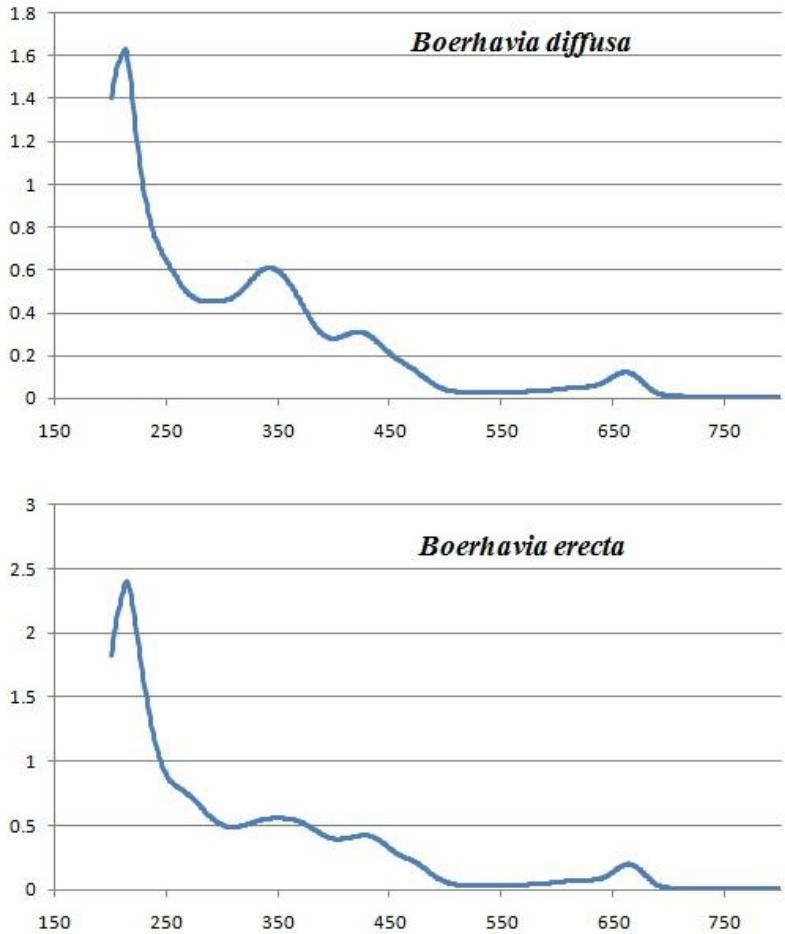
Figure 2: Total phenolic content of methanolic extract of above ground parts of *Boerhavia diffusa* and *boerhavia erecta*



3.2 Spectroscopic analysis

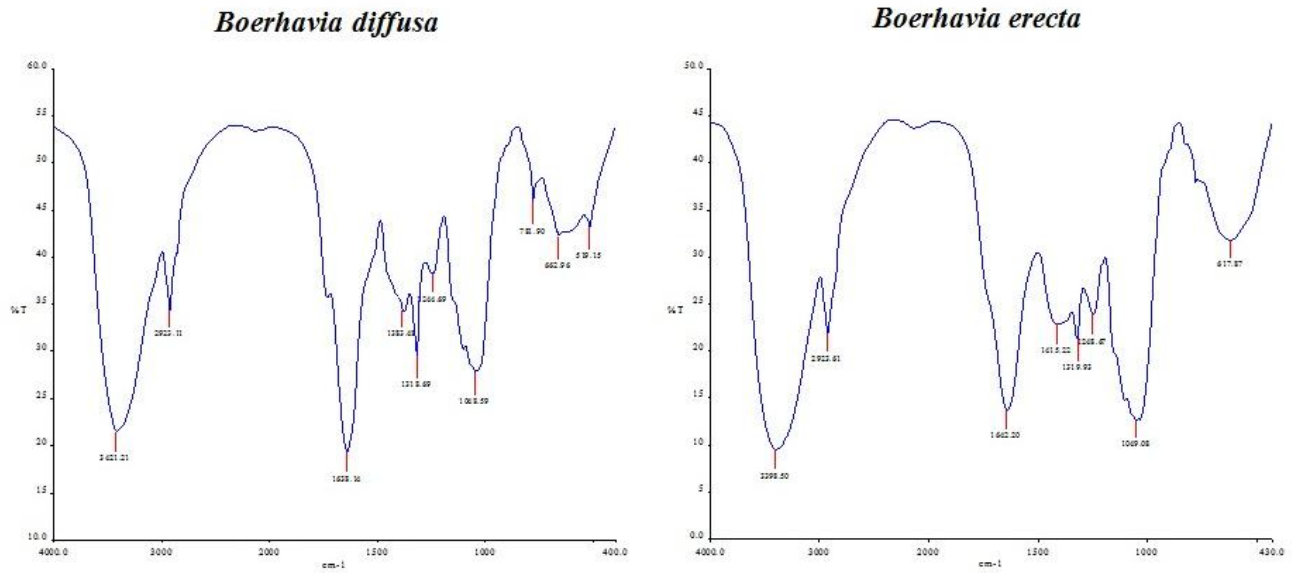
Suitably diluted methanolic extracts of *B. diffusa* and *B. erecta* revealed differences at UV-Visible spectrometer scanning (Fig. 3). The absorption of methanolic extract (30 folds diluted) of *B. diffusa* was 2.155 at 212 nm, 0.362 at 362 nm, 0.520 at 428 nm, 0.030 at 526 nm and 0.267 at 663 nm. Methanolic extract of *B. erecta* exhibited absorbance at 214 nm (2.404), 308 nm (0.485), 349 nm (0.555), 532 nm (0.027) and 663 nm (0.197). So, UV-Visible absorbance of methanolic extracts could be used as one of the quality control parameter to differentiate *B. diffusa* from *B. erecta*. Application of UV-Visible spectroscopy in determining the herbal fingerprints was explained in detail by Joshi [31]. UV-Vis spectroscopy has been applied to detecting the presence of extraneous food colourants [32].

Figure 3: UV-visible scanning of spectrum of methanolic extract of selected plant materials



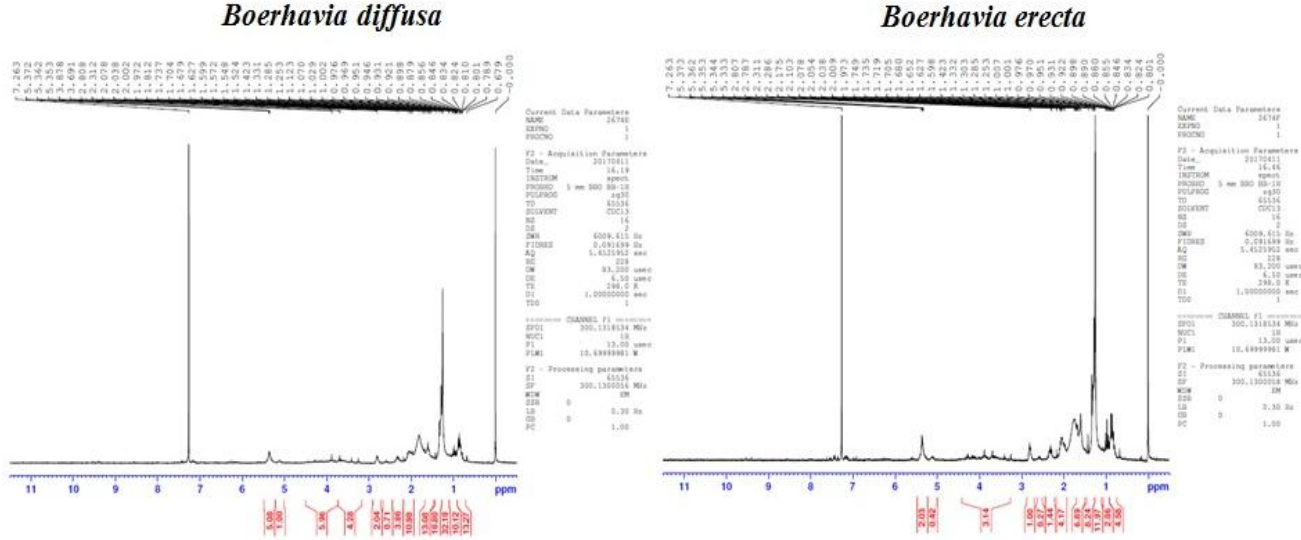
FT-IR spectroscopic analysis of finely powdered plant materials revealed significant difference at 1000 – 400 cm^{-1} region (Fig. 4). *B. diffusa* plant material exhibited transmission of 21.53, 34.43, 19.27, 34.28, 29.56, 38.23, 27.87, 45.87, 42.33 and 43.23% at 3421, 2923, 1638, 1383, 1318, 1244, 1048, 781, 662 and 519 cm^{-1} region, respectively. Raw material of *B. erecta* exhibited percentage of transmission of 9.51% at 3398 cm^{-1}, 21.86% at 2923 cm^{-1}, 13.75% at 1642 cm^{-1}, 22.91% at 1415 cm^{-1}, 21.28% at 1319 cm^{-1}, 23.96% at 1248 cm^{-1}, 12.65% at 1049 cm^{-1} and 31.76% at 617 cm^{-1}. Peaks at 2923, 1318, 1244 and 1048 cm^{-1} are common for both the plant samples, but peaks at 3421, 1638, 1383, 781, 662 and 519 cm^{-1} are unique for *B. diffusa*. Similarly, *B. erecta* exhibited unique peaks at 3398, 1642, 1415 and 617 cm^{-1}. Hence, these FT-IR profiles would be helpful to authenticate the selected herbals. Similarly, FT-IR technique was used to identify the adulterants of *Oregano vulgare* [33].

Figure 4: FT-IR spectrum of selected plant materials



NMR spectroscopy involves the analysis of the energy absorption by atomic nuclei with non-zero spins in the presence of a magnetic field. The energy absorptions of the atomic nuclei are affected by the nuclei of surrounding molecules, which cause small local modifications to the external magnetic field. NMR spectroscopy can therefore provide detailed information about the molecular structure of a food sample, given that the observed interactions of an individual atomic nucleus are dependent on the atoms surrounding it. NMR spectroscopic results of chloroform extracts of selected plant materials were shown in Fig. 5. Methanolic extract was used for all other analysis like phytochemical analysis, UV-Visible spectroscopy, HPLC and HPTLC, but it can't be analyzed in NMR and hence, chloroform extract was prepared exclusively for NMR analysis. NMR spectrum of *B. diffusa* showed signals at 5.08, 1.00, 5.96, 4.28, 2.04, 0.71, 3.86, 10.98, 13.08, 18.80, 32.18, 10.12 and 13.27 ppm. *B. erecta* showed signals at 2.03, 0.42, 3.14, 1.00, 0.27, 1.44, 4.17, 6.89, 8.24, 11.97, 2.86 and 4.58 ppm. Hence, such unique peaks could be used to differentiate *B. diffusa* from *B. erecta*. Similarly, Gilard et al. [34] and Vaysse et al. [35] have detected adulterants in herbal dietary supplements using H-NMR technique.

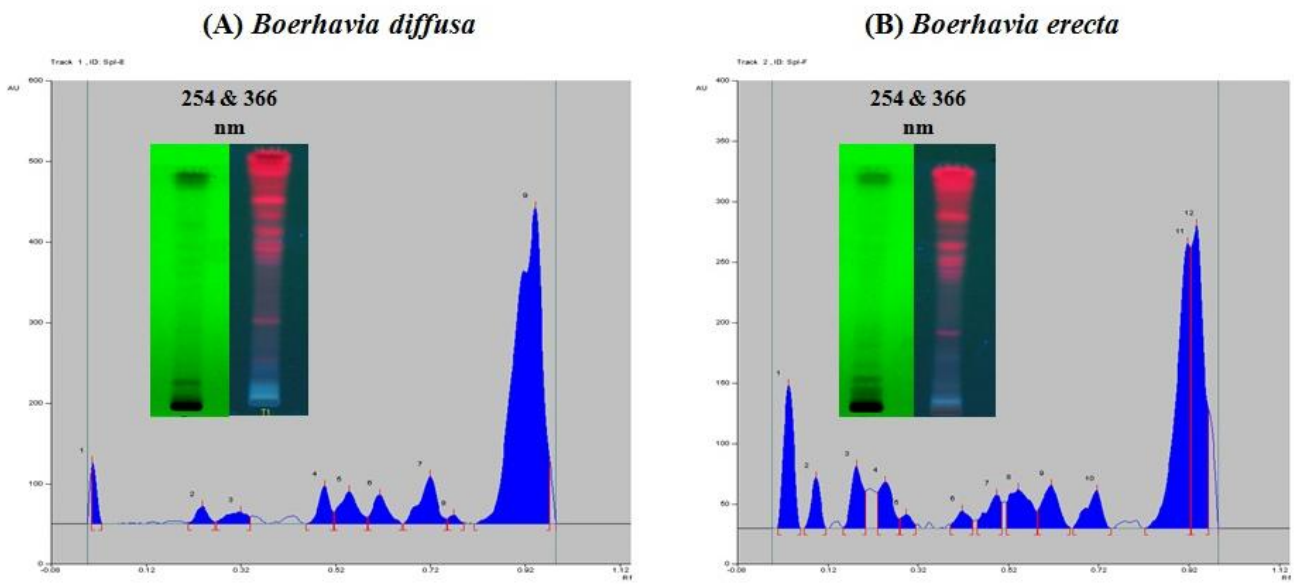
Figure 5: NMR spectrum of chloroform extract of selected plant materials



3.3 Chromatographic analysis

HPTLC is a very simple and rapid analytical method for high potential qualitative characterization and quantitative determination of herbals. Once chemical nature of phytoconstituents were established via HPTLC analysis, it is easy to standardize and validate the herbal products. HPTLC fingerprinting profile of methanolic extract of *B. diffusa* showed nine spots with the Rf value of 0.01, 0.24, 0.32, 0.50, 0.55, 0.62, 0.72, 0.77 and 0.95 (Fig. 6). But, twelve spots were observed in the case of *B. erecta* with the Rf value of 0.04, 0.10, 0.19, 0.25, 0.30, 0.42, 0.50, 0.55, 0.62, 0.72, 0.92 & 0.94. Among the detected spots, bands *B. diffusa* with the Rf value of 0.24, 0.32, 0.50. 0.55, 0.62, 0.72 and 0.95 were found to be comparable to that of *B. erecta*, which indicated the presence of similar type of compounds in both the plants. But, bands with Rf value of 0.01 and 0.77 are unique for *B. diffusa* while *B. erecta* exhibited unique spots with Rf value of 0.04, 0.10, 0.19, 0.42 and 0.92. These unique HPTLC profiles could be useful in distinguishing *B. diffusa* from *B. erecta*. Application of HPTLC fingerprints in determination of quality of botanicals was explained by Nicoletti [36]. Braz et al. [37] have used HPTLC technique to establish quality standards of selected plant species commonly found in the Brazilian market.

Figure 6: HPTLC profile of methanolic extract of selected plant materials

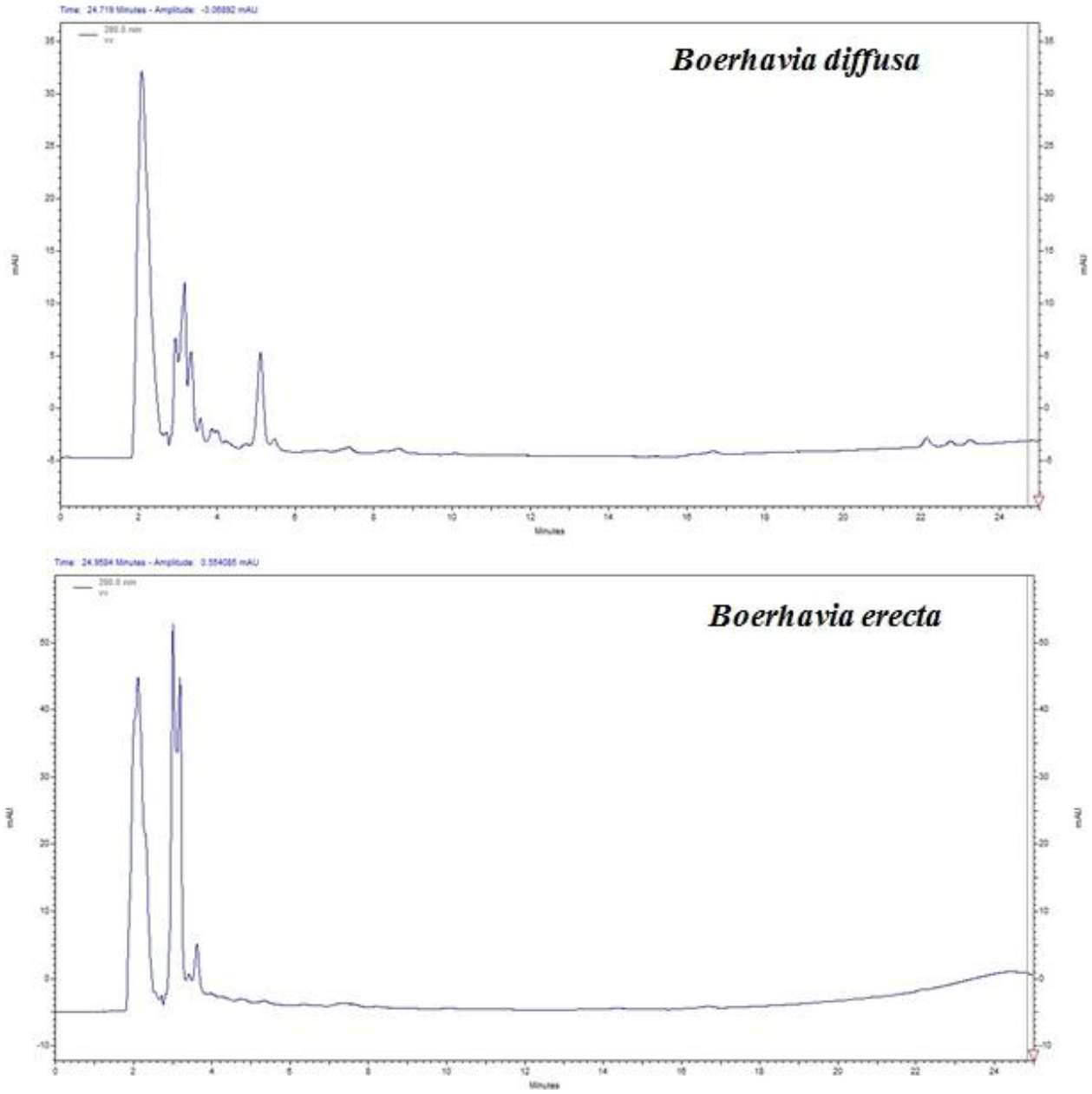


Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.01	66.7	0.01	75.9	10.88	0.03	0.8	637.8	2.84
2	0.21	1.2	0.24	21.6	3.09	0.27	2.2	401.6	1.79
3	0.27	2.9	0.32	14.8	2.13	0.34	8.9	496.8	2.21
4	0.46	0.3	0.50	47.1	6.75	0.52	14.6	799.6	3.56
5	0.52	14.8	0.55	40.1	5.75	0.59	8.3	1109.3	4.94
6	0.59	8.6	0.62	35.9	5.14	0.66	0.1	835.2	3.72
7	0.67	0.2	0.72	58.7	8.41	0.76	6.0	1548.7	6.89
8	0.76	6.3	0.77	10.9	1.56	0.79	1.5	166.1	0.74
9	0.82	0.1	0.95	392.6	56.29	0.98	80.9	16476.0	73.32

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.01	1.9	0.04	118.6	13.32	0.06	0.1	1958.9	10.34
2	0.07	0.2	0.10	42.0	4.71	0.12	0.2	611.1	3.22
3	0.16	1.0	0.19	51.7	5.81	0.21	30.7	1003.7	5.30
4	0.23	29.8	0.25	38.5	4.32	0.28	8.2	867.0	4.58
5	0.28	8.2	0.30	11.6	1.30	0.32	3.2	205.4	1.08
6	0.40	3.2	0.42	14.3	1.61	0.45	6.0	318.2	1.68
7	0.45	7.0	0.50	27.5	3.09	0.51	21.5	659.5	3.48
8	0.52	21.5	0.55	32.3	3.63	0.59	14.0	1163.0	6.14
9	0.59	14.6	0.62	35.6	4.00	0.66	0.9	957.7	5.05
10	0.67	0.5	0.72	31.5	3.54	0.75	0.0	801.1	4.23
11	0.83	0.3	0.92	235.8	26.49	0.93	232.1	5458.2	28.80
12	0.93	232.3	0.94	250.9	28.18	0.97	99.3	4946.7	26.10

HPLC is a popular method for the analysis of herbal medicines because of its easiness and HPLC analysis is not limited by the volatility or stability of the sample compound. In general, it can be used to analyze almost all the compounds in the herbal medicines. Thus, over the past decades, HPLC has received the most extensive application in the analysis of herbal medicines. HPLC fingerprinting data of methanolic extracts of *B. diffusa* and *B. erecta* were given in Fig. 7.

Figure 7: HPLC profile of methanolic extract of selected plant materials



B. diffusa extract showed a total number of 5 peaks with the retention time of 2.0, 3.0, 3.2, 3.4 & 5.0 min and among these, peak at 2.0 min has higher peak area. In the case of *B. erecta*, a total number of 4 peaks with retention time of 2.0, 3.0, 3.2 and 3.6 min were noticed and among which peak at 3.0 min was found to has higher percentage of peak area. Among the HPLC peaks, peak at 2.0, 3.0 & 3.2 min were common for both the plant samples, but peak at 3.4 & 5.0 min are unique for *B. diffusa* whereas *B. erecta* revealed unique peak at 3.6 min. So, these unique HPLC peaks could be useful in identification of drug material as either *B. diffusa* or *B. erecta*. Deconinck et al. [38] have developed HPLC methods to detect illegal pharmaceutical preparations. HPLC profile data of different basil species (*Ocimum americanum*, *O. basilicum*, *O. citriodorum* and *O. minimum*) were utilized for authentication purpose [39].

4. Conclusions

Identification of crude raw drug and authentication of proper herbal is the real challenge in herbal industry, because most of the herbal drugs are sold in powder form and sometimes adulterated with morphologically similar materials / related species plant parts. Hence, development of chemical fingerprints for the identification of proper herbal drug and also to differentiate the adulterants is of paramount importance in herbal industry. In this context,

we have developed chemical fingerprints of *B. diffusa* and its adulterant *B. erecta* using phytochemical analysis, spectroscopic methods (UV-Visible, FT-IR and NMR) and chromatographic techniques (HPTLC & HPLC). Results obtained from the present work indicated that the chemical fingerprints might be useful in differentiating *B. diffusa* from *B. erecta*. Experimental results could be useful in quality control process and also to detect the use *B. diffusa* or *B. erecta* in herbal treatment.

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