Bulletin of Pharmaceutical Research 2017;7(2):147
An Official Publication of Association of Pharmacy Professionals
ISSN: 2249-6041 (Print); ISSN: 2249-9245 (Online)
DOI: 10.21276/bpr.2017.7.2.5

RESEARCH PAPER

DEVELOPMENT OF CHEMICAL FINGERPRINTS TO DIFFERENTIATE THE CONTROVERSIAL INDIAN HERBAL DRUGS *ECLIPTA PROSTRATA* AND *WEDELIA CALENDULACEA* (*BHRINGRAJA*)

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Received: Jun 08, 2017 / Revised: Aug 01, 2017 / Accepted: Aug 02, 2017

In Indian system of medicine, *Eclipta prostrata* is an important herbal ingredient of several Ayurvedic and Siddha formulations and it is commonly called as *Bhringraja*. But, this plant is often confused with *Wedelia calendulacea*, which is also known as *Bhringraja* and also being adulterated in Indian herbal market. In order to differentiate these plant drugs (*Eclipta prostrata* and *Wedelia calendulacea*), in the present work we have developed chemical fingerprints. Phytochemical analysis, UV-Visible, FT-IR and NMR spectroscopy, HPTLC and HPLC chromatography were carried out in the selected plant materials. Phytochemical analysis of methanolic extract of investigated materials showed that *W. calendulacea* contained higher level of total phenolic compounds (381.82 mg GAE/100 g) when compared to *E. prostrata* (105 mg GAE/100 g). Scanning in UV-Visible spectroscopy exhibited difference between selected samples, especially at 250-450 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 1-2 ppm region. HPTLC profile of methanolic extract of *E. prostrata* exhibited a total number of 9 peaks while *W. calendulacea* showed 12 number of peaks. Chemical profile investigated in the present study would be helpful to differentiate *E. prostrata* from *W. calendulacea* and also useful to prevent their adulteration in herbal industry.

Key words: Bhringraja, *Eclipta prostrata*, *Wedelia calendulacea*, Herbal adulteration.

INTRODUCTION

Quality issues including the genuineness of crude drugs are a major concern plaguing the herbal drug industry particularly in India. The use of authentic medicinal plant species is a fundamental requirement in herbal medicine (Govindaraghavan *et al* 2012). Adulteration, substitution, and mislabelling may reduce the efficacy of the herbal drugs and also cause potential side effects to the patients (Newmaster *et al* 2013). Literature is enriched with diverse reports with bioactivities of herbal constituents (Jain *et al* 2011; Chowdhury *et al* 2012; Deb *et al* 2013; Rashid *et al* 2014; Agarwal *et al* 2015; Shrestha *et al* 2016; Viana *et al* 2017) but, along with safety and toxicity, there is a need to develop methods for herbal authentication. Correct botanical identity of several crude drugs

used in Indian traditional systems has not been properly established (Dixit, 2011). One of the examples for such controversial herbal drugs is *Bhringraja*. Both *prostrata* and *Wedelia calendulacea* are commonly called as *Bhringraja* and reports about *Eclipta* and their frequent misuses (Pendkar *et al* 2016).

Eclipta prostrata (Syn.: *E. alba*, Tamil: *Karisalai*, Family: Asteraceae) is traditionally used as a liver tonic and hair growth promoter in Indian traditional systems of medicine. It is used in the treatment of spleen enlargements, uterine hemorrhages, skin diseases, scorpion bites and respiratory disorders (Pithayanukul *et al* 2004; Khare, 2007). This plant has been experimentally proven to possesses nephro-protective, cerebroprotective, hepato-protective, anti-cancer, anti-asthma, mosquito larvicidal and ovicidal properties, toxicity against hepatitis-C virus, hair growth promoting activity, immune-modulatory property, antioxidant, lipid lowering activity and anti-venom property (Dungca, 2016; Chaudhary *et al* 2011; de Freitas Morel *et al* 2017; Jayathirtha and Mishra, 2004; Datta *et al* 2009; Govindarajan and Karuppannan, 2011). Presence of wedelolactone, ecliptamines, taraxastane glycosides, polythienyl and other phytochemicals has been reported in this plant (Zhang *et al* 2010; Liu *et al* 2012; Han *et al* 2013).

Wedelia calendulacea (L.) (Syn.: *W. chinensis*, Tamil: *Manjal karisalai*) also belongs to the family Asteraceae and has a similar common name, *Bhringraja*. It is used in Ayurveda, Sidhha and Unani systems of medicine to cure jaundice, uterine haemorrhage, menorrhagia, cough, cephalagia, dyeing grey hair, promoting hair growth, treat skin problems, dermatitis, eczema, hepato-protective, anti-inflammatory activity, anti-microbial, anti-osteoporosis, neuro-sedative and anti-cancer (Annie *et al* 2006; Lin *et al* 2007). Phytochemicals like indole-3-carboxyl-aldehyde, wedelolactone, kauren diterpenes, luteolin, apigenin were reported (Haider *et al* 2003).

W. calendulacea is often used as an adulterant / substitute for *E. prostrata* due to the confusion in their common name, *Bhringraja*. A comparative study on the pharmacognostic parameters of both *E. alba* and *W. calendulaceae* is reported in literature (Gopalakrishnan and Solomon, 1992). Recently, ISSR and RAPD-based molecular markers have been developed (Pendkar *et al* 2016) to differentiate *E. alba* and *W. calendulacea*. In this connection, in the present study, we have made an attempt to develop chemical fingerprints to differentiate *E. prostrata* from its adulterant *W. calendulacea* by using modern analytical tools.

MATERIALS AND METHODS

Sample preparation

Above-ground parts of *E. prostrata* was collected from SASTRA herbal garden, Thachenkuruchi, Thanjavur and *W. calendulacea* was collected from a private herbal garden, Modakurichi, Erode, Tamilnadu, India. 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.

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-IR and HPTLC finger-printing.

Quantification of polyphenols

Total phenolic content of methanolic extract of *E. prostrata* and *W. calendulacea* were analyzed using Folin-Ciocalteu reagent method with some modifications (Singleton *et al* 1998). 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 was 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).

Spectroscopic analysis

UV-Visible scanning of suitably diluted methanolic extract of *Eclipta prostrata* and *Wedelia calendulacea* 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 *E. prostrata* and *W. calendulacea* were oven dried at 60°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 ^{1}H NMR spectra of chloroform extract of *E. prostrata* and *W. calendulacea* (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.

Chromatographic analysis

HPTLC profiles of methanolic extract of *E. prostrata* and *W. calendulacea* were analyzed on a pre-coated silica gel 60 F-254 (0.2 mm thickness) HPTLC plate (10 × 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 R_f values and fingerprint data were recorded using WINCATS software.

For HPLC analysis, methanolic extract of *E. prostrata* and *W. calendulacea* 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 × 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.

RESULTS AND DISCUSSION

Both *E. prostrata* and *W. calendulacea* has many morphological similarities, but can be distinguished using botanical characters. *E. propstrata* is a small herb with soft, branched stem and leaves are opposite, sessile, lanceolate and serrated margin (**Figure 1**).

Fig. 1. Morphology of selected plants *E. prostrata* (A), *W. calendulacea* (B) and their powdered samples (C, D)

Roots are cylindrical with greyish colour. The solitary flower heads are 6 - 8 mm in diameter with white florets. The achenes are compressed and narrowly winged. *W. calendulacea* is a small, highly branched annual herb shows rooting at nodes. Leaves are opposite, sub-sessile, lanceotate-oblong, entire or irregularity crenate and serrate. Both the surfaces of leaves are covered with sharp-pointed stiff hairs. Heads are bright yellow coloured, stalked, about 1 cm diameter, solitary, oblong with axillary peduncles. The ray florets are 8-12 in number, spreading, about equal to the bracts and broad; the disk florets are 20 in number, short, narrow and pointed. Achenes are nearly cylindrical, acute and pubescent. Major difference between these plants is the colour of the flowers: White

colour in the case of *E. prostrata* and bright yellow flowers in *W. calendulacea*. 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. Hence, based on the availability, location and season, the commercial vendors could collect either *E. prostrata* or *W. calendulacea* and sell in the market as *Bhringaraja* in dried powder form. In this context, development of chemical fingerprints is necessary for quality control of *Bhringaraja* drug in Indian herbal industry and also to authenticate the proper drug.

Polyphenolic content

Analysis of total phenolic content of methanolic extract of selected plant materials revealed that *W. calendulacea* contained higher level of total phenolic compounds (381.82 mg GAE / 100 g) when compared to *E. prostrata* (105 mg GAE / 100 g) (**Figure 2**). Higher content of polyphenols observed in methanolic extract of *W. calendulacea* of the present analysis provides scientific evidence for the better efficacy noted in traditional medicine.

However, it is considered as adulterant for the proper drug *E. prostrata* in Indian system of medicine. In a previous study, higher level of total phenolic content of *E. prostrata* (2250 mg GAE / 100 g) was reported when compared to the results of present study, which might be due to the ultrasonic-assisted extraction process (Fang *et al* 2014). Quantification of total phenolic compounds using Folin's-Ciocaltue reagent (Spectrometric method) could help us to differentiate *E. prostrata* from *W. calendulacea*.

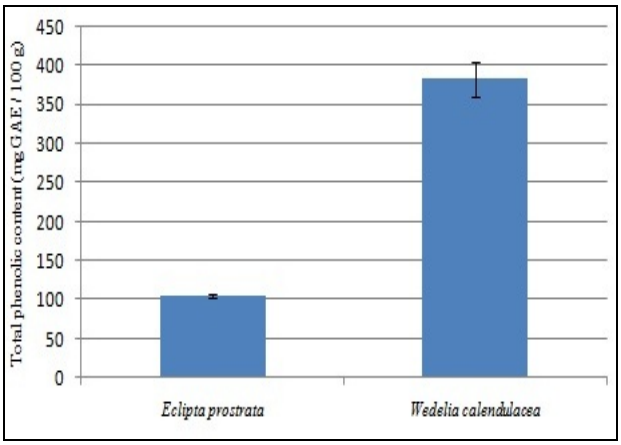


Fig. 2. Total phenolic content of methanolic extracts of ground parts of *E. prostrata* and *W. calendulacea*

Spectroscopic analysis

Suitably diluted methanolic extracts of *E. prostrata* and *W. calendulacea* revealed differences at UV-Visible spectrometer scanning, especially at 250-450 nm region (**Figure 3**). The absorption of methanolic extract (30 folds diluted) of *W. calendulacea* was 2.79 at 250 nm, 2.099 at 300 nm, 1.53 at 350 nm, 0.749 at 400 nm and 0.501 at 450 nm, which are higher when compared to the absorbance of *E. prostrata* at 250 nm (1.707), 300 nm (1.216), 350 nm (0.919), 400 nm (0.473) and 450 nm (0.308). So, UV-Visible absorbance of methanolic extract could be used as one of the quality control parameter. Application of UV-Visible spectroscopy in determining the herbal fingerprints is reported in literature (Joshi, 2012). UV-Vis spectroscopy has been applied for detecting the presence of extraneous food colorants (Gonzalez *et al* 2005).

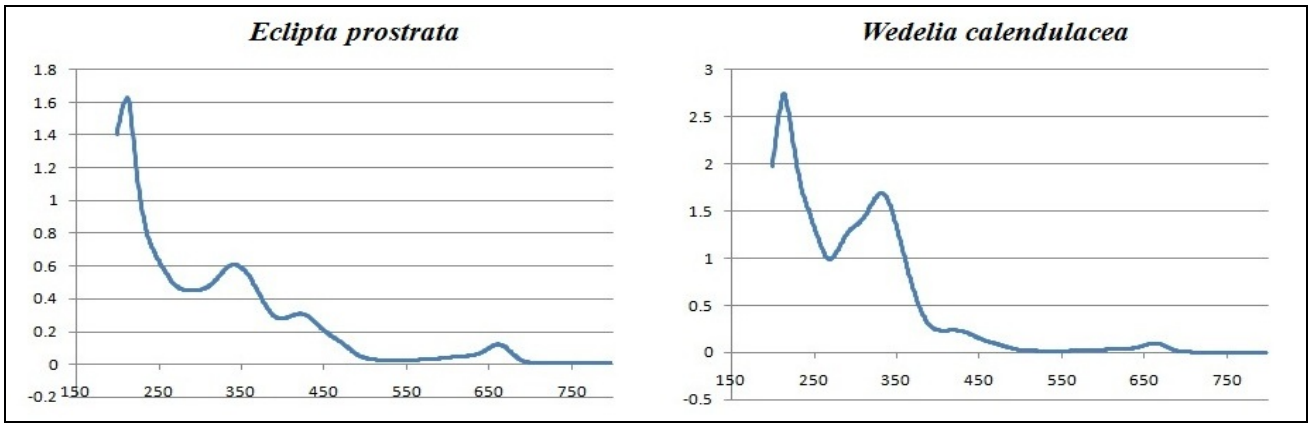


Fig. 3. UV-Visible scanning spectrum of ethanolic extracts of selected plant materials

FT-IR spectroscopic analysis of finely powdered plant materials revealed significant difference at 1000 – 400 cm^{-1} region (**Figure 4**). *E. prostrata* plant material exhibited transmission of 15.90, 29.20, 21.19, 30.89, 33.70, 29.58, 21.32, 41.99, and 46.26% at 3395, 2925, 1634, 1423, 1324,

1255, 1055, 599, and 470 cm^{-1} region, respectively.

Raw material of *W. calendulacea* exhibited percentage of transmission of 22.48% at 3394 cm^{-1}, 35.11% at 2922 cm^{-1}, 26.40% at 1638 cm^{-1}, 34.04% at 1422 cm^{-1}, 35.08% at 1243 cm^{-1}, 23.48% at 1052 cm^{-1}, 46.08% at 776 cm^{-1}, 43.98% at 592 cm^{-1} and 43.40% at 468 cm^{-1}. Peak at 1324 cm^{-1} is unique for *E. prostrata*, which is not available in *W. calendulacea*. Similarly, *W. calendulacea* exhibited unique peak at 776 cm^{-1}, which is not found in *E. prostrata*. 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* (Black *et al* 2016).

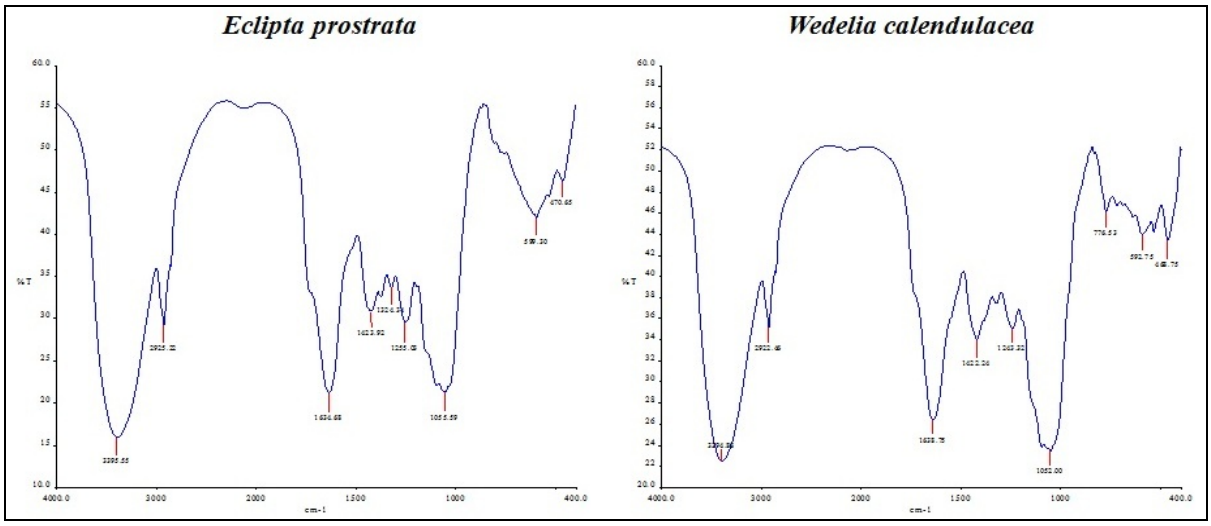


Fig. 4. FT-IR spectrum of methanolic extracts of selected plant materials

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 **Figure 5**.

Methanolic extract was used for all other analysis like phytochemical analysis, UV-Visible spectroscopy, HPLC and HPTLC, but it cannot be analyzed in NMR and hence, chloroform extract was prepared exclusively for NMR analysis. NMR spectrum of *E. prostrata* showed three major peaks at 1-2 ppm region whereas only one major peak was observed in the case of *W. calendulacea* in that region. The peak at 1.3 ppm region is common for both the selected plant samples, but peaks noted at 1.7 and 2.0 ppm are unique for *E. prostrata*. Hence, such unique peaks could be used to differentiate *E. prostrata* from *W. calendulacea*. Similarly, literature is enriched with reports indicating detection of adulterants in herbal dietary supplements using ^{1}H NMR technique (Gilard *et al* 2015; Vaysse *et al* 2010).

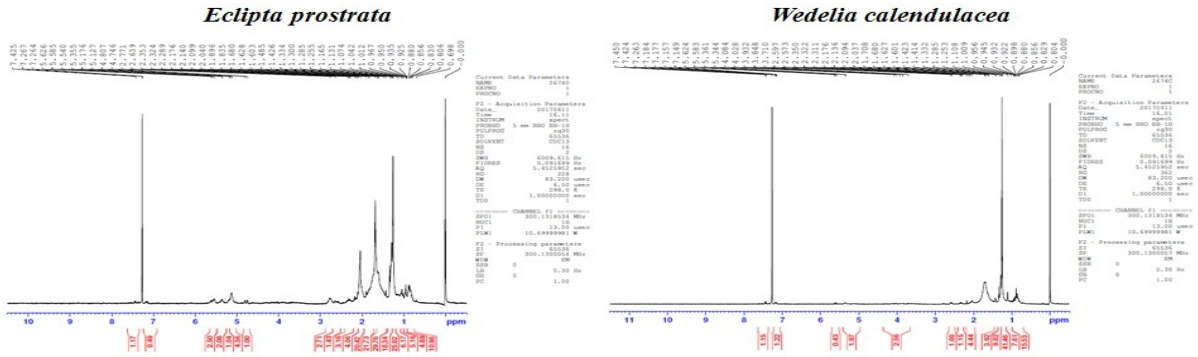
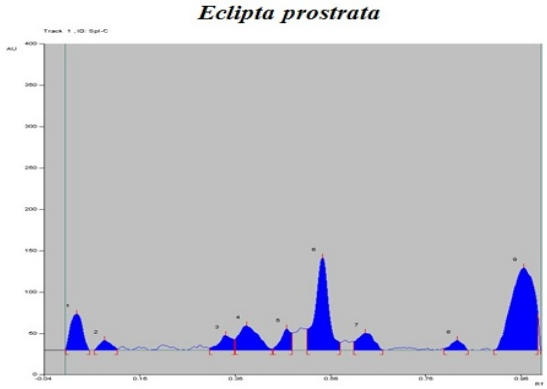


Fig. 5. NMR spectrum of chloroform extracts of selected plant materials

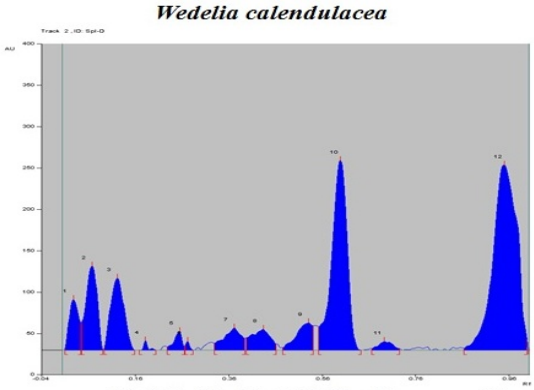
Chromatographic analysis

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 *E. prostrata* showed nine spots with the R_f value of 0.02, 0.08, 0.34, 0.38, 0.46, 0.54, 0.63, 0.82 and 0.96 (**Figure 6**).

But, twelve spots were observed in the case of *W. calendulacea* with the R_f value of 0.02, 0.06, 0.12, 0.18, 0.25, 0.27, 0.37, 0.43, 0.53, 0.60, 0.69 & 0.95. Among the detected spots, bands *E. prostrata* at R_f value of 0.02, 0.08, 0.38. 0.46, 0.54, 0.63 and 0.96 were found to be comparable to that of *W. calendulacea*, which indicated the presence of similar type of compounds in both the plants. But, bands with R_f value of 0.34 and 0.82 are unique for *E. prostrata* while *W. calendulacea* exhibited unique spots with R_f value of 0.12, 0.18, 0.25, 0.27 and 0.69. These unique HPTLC profiles could be useful in distinguishing *E. prostrata* from *W. calendulacea*. Application of HPTLC fingerprints in determination of quality of botanicals employed HPTLC technique to establish quality standards of selected plant species commonly found in the Brazilian market (Nicoletti, 2011; Braz *et al* 2012).



Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.00	2.3	0.02	43.8	11.75	0.05	0.3	850.4	8.89
2	0.06	0.6	0.08	11.9	3.19	0.11	1.5	221.6	2.32
3	0.30	2.6	0.34	17.6	4.73	0.36	12.7	387.2	4.05
4	0.36	12.9	0.38	29.6	7.95	0.43	1.4	923.8	9.66
5	0.44	1.7	0.46	26.1	7.00	0.48	21.0	426.2	4.46
6	0.51	25.7	0.54	111.3	29.88	0.58	9.5	2307.1	24.12
7	0.61	10.5	0.63	20.7	5.55	0.67	0.5	546.9	5.72
8	0.80	2.4	0.82	11.9	3.21	0.85	0.2	226.5	2.37
9	0.90	0.6	0.96	99.6	26.73	0.99	44.2	3674.9	38.42



Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.01	1.3	0.02	61.2	7.25	0.04	33.1	976.5	4.31
2	0.04	33.3	0.06	101.7	12.05	0.09	0.7	1902.0	8.40
3	0.09	1.1	0.12	87.1	10.32	0.16	0.0	1938.4	8.56
4	0.16	0.0	0.18	11.5	1.36	0.20	0.0	71.6	0.32
5	0.22	4.0	0.25	22.5	2.66	0.26	4.6	290.1	1.28
6	0.26	5.4	0.27	10.7	1.27	0.28	0.1	67.7	0.30
7	0.33	8.5	0.37	26.9	3.19	0.39	14.6	785.3	3.47
8	0.39	14.8	0.43	25.6	3.03	0.46	7.8	811.0	3.58
9	0.47	3.1	0.53	33.2	3.94	0.54	29.7	903.2	3.99
10	0.55	28.5	0.60	229.3	27.16	0.64	0.2	5822.0	25.71
11	0.66	1.9	0.69	10.6	1.25	0.72	1.9	258.7	1.14
12	0.86	2.3	0.95	223.9	26.52	1.00	12.9	8819.2	38.94

Fig. 6. HPTLC profiles of methanolic extracts of selected plant materials

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 herbal medicinal compounds. 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 *E. prostrata* and *W. calendulacea* was given in **Figure 7**. *E. prostrata* extract showed a total number of 10 peaks with the retention time of 1.9, 2.1, 3.0, 3.2, 3.6, 4.0, 4.4, 5.4, 8.0 & 10.0 and among these, peak at 4.0 min has higher peak area. In the case of *W. calendulacea*, a total number of 10 peaks with retention time of 1.9, 2.0, 2.1, 3.0, 3.2, 3.6, 4.0, 5.4, 7.0 and 10.0 were noticed and among which peak at 4.0 min was found to has higher percentage of peak area.

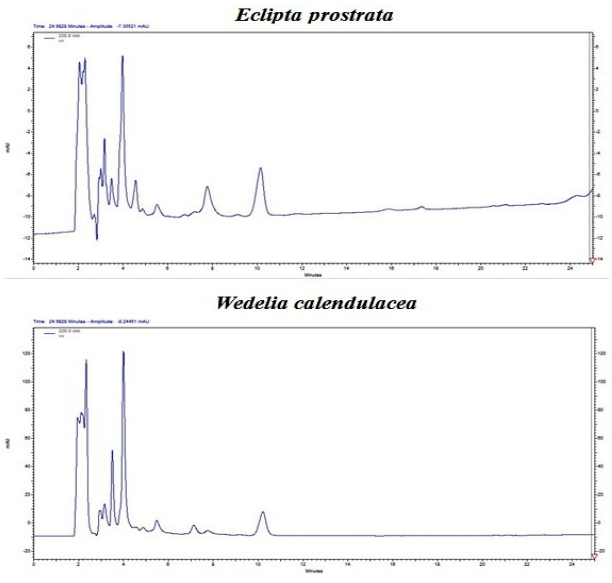


Fig. 7. HPLC profile of methanolic extracts of the selected plant materials

Among the HPLC peaks, peak at 4.4 and 8.0 min were unique for *E. prostrata* whereas *W. calendulacea* revealed unique peaks at 2.0 and 7.0 min. So, these unique HPLC peaks could be useful in identification of drug material as either *E. prostrata* or *W. calendulacea*. In literature, there are reports indicating development of HPLC methods to detect illegal pharmaceutical preparations (Deconinck *et al* 2013). HPLC profile data of different basil species (*Ocimum americanum*, *O. basilicum*, *O. citriodorum* and *O. minimum*) were utilized for authentication purpose (Grayer *et al* 2004).

CONCLUSION

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

ACKNOWLEDGEMENTS

One of the authors (VV) is thankful to National Medicinal Plant Board (NMPB), Ministry of AYUSH, New Delhi for given the financial support (Grant No. Z. 18017/187/CSS/R&D/TN-03/2016-17-NMPB-IV-A) and authors are thankful to the Honourable Vice-Chancellor of SASTRA University, Thanjavur, Tamilnadu for his encouragement and constant support to conduct this research project.

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